Study of novel molecular defects in human pancreas dysfunction

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ABSTRACT

Diabetes is a worldwide health problem caused by the loss or dysfunction of the insulin-secreting β-cells in the pancreas. Despite extensive research, fundamental processes during pancreas development remain to be determined, hampering our understanding of the disease mechanisms. Unelucidated forms of monogenic diabetes, arising from rare mutations in one single gene, represent invaluable models for identifying new targets of β-cell development and function.

In this study, I focused on putative disease-associated genes for diabetes that have been previously identified by next-generation sequencing of a cohort of patients with puberty-onset diabetes. In particular, I investigated unique mutant variants in genes coding for Histone deacetylase 4 (HDAC4), Glioma-associated oncogene homolog 1 (GLI1) and Glioma-associated oncogene homolog 2 (GLI2). These transcriptional regulators were prioritized for functional analysis based on patient phenotype, expression level in pancreas progenitor cells and available genetic information. To investigate the role of the genetic mutant variants in pancreatic cell fate decisions and cell function, I used the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 genome editing technology in combination with human induced pluripotent stem cell (iPSC)-directed β-cell differentiation. Employing these approaches, I established several patient-like iPSC lines carrying the identified heterozygous missense variants. Specifically, functional experiments and whole transcriptome analysis showed that the variant c.C4661T in *GLI2* impairs human β-cell differentiation and β-cell function, which might be responsible for a genetic predisposition to develop diabetes. In addition, I used the same iPSC-based differentiation model system to study novel extrinsic factors, namely the HDAC inhibitor HC toxin and the ROBO ligand SLIT3 and uncovered their conserved role in enhancing human β-cell development.

Taking together, I established a human iPSC differentiation platform to study critical genes and extrinsic factors that are necessary for human pancreas development and/or β-cells. This approach has provided new insights into the pathogenesis and therapeutic approaches for diabetes.

Keywords: Pancreas, Diabetes, Development, β-cells, iPSCs, CRISPR-Cas9

ZUSAMMENFASSUNG

Diabetes ist ein weltweites Gesundheitsproblem, das durch den Verlust oder die Dysfunktion der Insulin-produzierenden β-Zellen der Bauchspeicheldrüse verursacht wird. Trotz zahlreicher Studien sind grundlegende Prozesse der Entwicklung dieses Organs unbekannt. In seltenen Fällen entsteht Diabetes durch eine Mutation in einem einzigen Gen. Diese sogenannten monogenetischen Formen des Diabetes können zur Identifizierung neuer Regulatoren der β-Zellen-Entwicklung und -Funktion beitragen.

Im Fokus der vorliegenden Arbeit habe ich neue putative Diabetes-assoziierte Gene untersucht, die zuvor durch „Next-Generation“ Sequenzierung in einer Gruppe von Kindern und Jugendlichen mit idiopathischem Diabetes festgestellt wurden. Insbesondere analysierte ich neuartige Mutationsvarianten in Genen kodierend für Histone deacetylase 4 (HDAC4), Glioma-associated oncogene homolog 1 (GLI1) und Glioma-associated oncogene homolog 2 (GLI2). Basierend auf den folgenden Kriterien wurden diese Transkriptionsregulatoren zur weiteren funktionellen Analyse priorisiert: Genetische Information, Patientenphänotyp und Expressionsprofil der Kandidaten Gene in Mauspankreas-Vorläuferzellen. Um die putative Rolle der Mutationsvarianten während der pankreatischen Zelltypspezifizierung zu untersuchen, nutzte ich die CRISPR-Cas9 Methode in Kombination mit Stammzellendifferenzierung. Im Detail generierte ich verschiedene Stammzellen mittels CRISPR-Cas9, die die neu entdeckten Mutationsvarianten der Patienten beherbergten und differenzierte diese zu β-ähnlichen Zellen. Weitere *in vitro* und Transkriptionsanalysen zeigten, dass die Patientenvariante c.C4661T in GLI2 die Entwicklung der β-ähnlichen Zellen beeinträchtigte, was für eine genetische Prädisposition zur Entwicklung einer Diabetes-Erkrankung verantwortlich sein kann. Zusätzlich nutzte ich diese Plattform, um neue extrinsische Faktoren zu untersuchen und zeigte, dass die fördernde Rolle von HC toxin (HDAC Inhibitor) und SLIT3 (ROBO Ligand) konserviert ist.

Zusammenfassend habe ich eine Differenzierungsplattform etabliert, um die Rolle von genetischen und extrinsischen Faktoren für die Entwicklung des Pankreas und/oder β-Zellen zu untersuchen. Dieser Ansatz lieferte neue Einblicke in die molekularen Mechanismen, die für die Entwicklung und Physiologie von β-Zellen relevant sind.

Stichworte: Pankreas, Diabetes, Entwicklung, β-Zellen, iPSCs, CRISPR-Cas9

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1 INTRODUCTION

1.1 The Pancreas

1.1.1 Architecture and function of the pancreas

The adult pancreas is a gland composed of both endocrine and exocrine tissues, which exert important metabolic functions [1–3]. The exocrine pancreas consists of acinar and ductal cells that are organized in a branched, tubular epithelial tree-like network. Acinar cells produce and secrete a variety of digestive enzymes, such as trypsin, chymotrypsin, and amylase. These enzymes are released as zymogens (proenzymes or inactive precursors of enzymes) and transported through the pancreatic ductal system to the duodenum, where they assist nutrient digestion. Besides transporting pancreatic zymogens, ductal cells also secrete bicarbonate to neutralize gastric acid in the duodenum [4]. On the other hand, the hormone-secreting endocrine cells cluster together to form discrete pancreatic islets, also known as islets of Langerhans, and regulate glucose homeostasis. Each of the highly vascularized pancreatic islets is composed of multiple endocrine cell types. These include the glucagon-expressing α-cells, the insulin-producing β-cells, the somatostatin-secreting δ-cells, the pancreatic polypeptide-producing PP-cells and the ghrelin-producing ε-cells [5,6]. It is estimated that the adult human pancreas contains about one million islets, each one harbouring roughly one billion insulin-secreting β-cells which contribute to 60-70% of the islet mass [7]. In mice islets display a very characteristic organization, with the β-cells located in the core surrounded by a mantle of α- and δ-cells. In humans, instead, islet architecture is more complex but still conforms to the overall structure of β-cells in the islet core, which are then intermingled with α-cells [8]. Pancreatic α- and β-cells are the most abundant and disease-relevant endocrine cells. Hormones secreted by these cells maintain blood glucose levels within a very narrow physiological range [9]. This is accomplished by the opposing and balanced actions of glucagon and insulin. Glucagon, secreted from the α-cells, stimulates hepatic glycogenolysis and gluconeogenesis and, thus, increases blood glucose levels. By contrast, insulin decreases blood glucose levels by promoting hepatic glycogenesis and glucose uptake in muscle and adipose tissues [9–11].

The pancreas is a target of several still incurable diseases, the most notable being pancreatitis, pancreatic adenocarcinoma and diabetes mellitus (see section 1.2) [12,13].

Moreover, unlike its embryonic close relative the liver, the pancreas shows limited tissue regeneration upon injury and disease [14,15]. Therefore, intensive efforts are underway to develop cell-replacement therapies [14,16,17]. Regardless of the strategy explored to replace lost and/or dysfunctional pancreatic cells, a profound understanding of the mechanisms driving pancreatic development and lineage specification are needed [16,18,19]. In line with this, the discovery of novel genes and pathways is of great value to further refine and optimize current cell-therapy approaches [20].

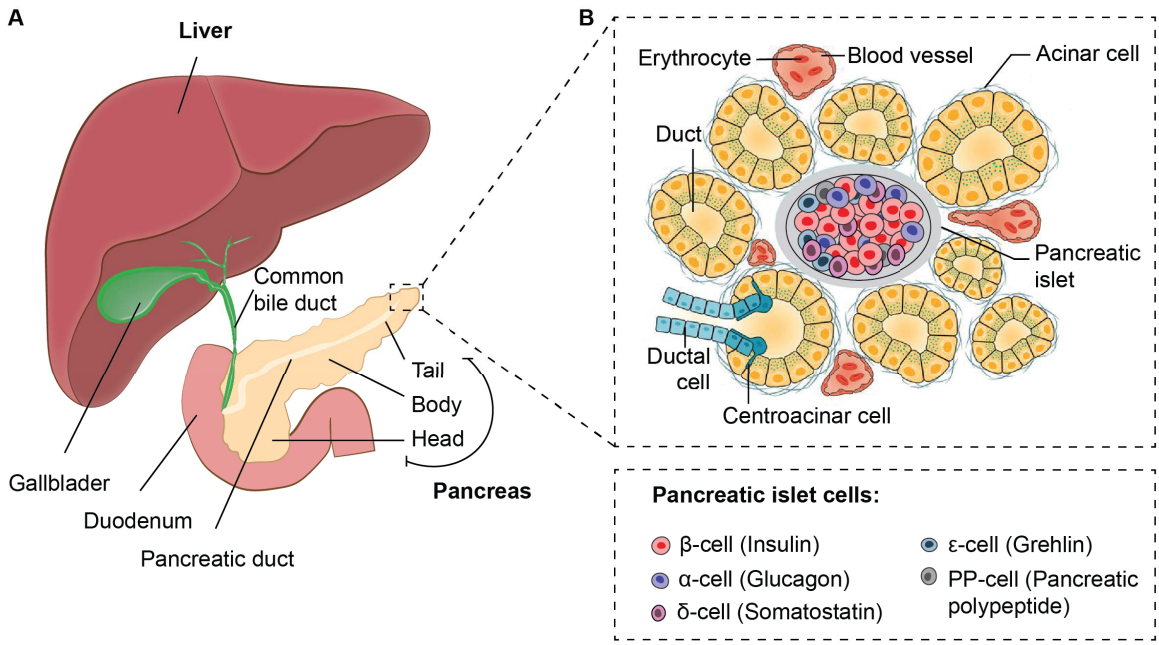

Fig. 1: The adult human pancreas. (**A**) Representation of the adult human pancreas. The pancreas is located behind the stomach, connected to the duodenum and adjacent to the spleen and liver. The human pancreas is morphologically divided into head, body and tail, and connected to the duodenum via the pancreatic duct. (**B**) Histological view of the pancreas shows the exocrine and endocrine compartments (acinar cells and islets of Langerhans, respectively) and the ductal network. Adapted from Ellis, C. et al. (2017).

1.1.2 Embryonic development of the pancreas and β-cells

During embryogenesis, cells undergo an array of lineage decisions to acquire their final identity [21,22]. In mammalian species, following fertilization, the zygote undergoes a series of cell divisions (i.e. cleavage) that result in the formation of the blastocyst [2,23]. The blastocyst encompasses the trophectoderm and the inner cell mass. After implantation the three germ layers–endoderm, mesoderm and ectoderm–form from the pluripotent epiblast layer of the inner cell mass, which ultimately give rise to every tissue of the body [24–26]. The pancreas, together with the liver, biliary system, lungs, thyroid, thymus, and epithelial lining of the respiratory and digestive system, derives from the endoderm [24–27]. Given that most

studies to date have been performed in mice and key mechanisms are conserved across species, the following sections will summarize fundamental events of pancreatic development in the mouse embryo.

1.1.2.1 Endoderm patterning and specification of the pancreas

During gastrulation, the definitive endoderm (DE) emerges from the anterior end of the primitive streak [21,25,27]. The DE undergoes a series of morphogenetic changes that ultimately lead to the formation of the primitive gut tube surrounded by mesoderm [2]. The commitment to either endoderm or mesoderm is modulated by a gradient of Nodal signalling, which belongs to the transforming growth factor beta (TGFβ) family [28–30]. Specifically, high Nodal is present in the anterior region of the primitive streak and promotes expression of genes required for establishing and maintaining an endodermal identity. These factors are the transcription factors (TFs) Eomesodermin (EOMES), Forkhead box protein A2 (FOXA2), Sex-determining region Y- box 17 (SOX17) and members of the GATA family [28,30,31]. Contrary, low Nodal induces formation of the mesoderm through activation of Fibroblast Growth Factor (FGF) and Brachury [32–34].

Throughout the process of gut tube morphogenesis, the endoderm is being patterned along the anterior-posterior (A-P) and dorso-ventral (D-V) axes [35]. Multiple signalling pathways play a role in regionalizing the endoderm into fore-, mid- and hindgut. Mesoderm-derived factors, such as Retinoic Acid, Transforming Growth Factor Beta / Bone Morphogenetic Protein (TGFβ/ BMP), FGF4 and Wingless-type MMTV integration site family (WNT) ligands provide lineage-inductive cues for mid- and hindgut [34,36–39]. In contrast, WNT antagonists secreted by the anterior endoderm promote a foregut identity. The foregut, marked by the expression of Hematopoietically expressed homeobox (*Hhex*), SYR-box 2 (*Sox2*) and *FoxA2*, will give rise to gastrointestinal structures, like the stomach, as well as to many endoderm-derived organs, such as liver and pancreas [40–42]. In detail, the foregut is competent to respond to inductive pancreatic signals from the notochord, endothelium and surrounding mesenchyme [3,26,43]. These signals induce the formation of a dorsal pancreatic bud, shortly later followed by the appearance of a ventral bud from the foregut endoderm. The ventral pancreatic region is specified in a domain adjacent to the pre-hepatic region and in close proximity to the cardiac mesoderm and septum transversum mesenchyme. Specification of the pancreatic endoderm is characterized by the expression of *Pancreatic and duodenal homeobox 1* (*Pdx1*) and Pancreas specific transcription factor 1a (*Ptf1a*) in the two rudiments. This occurs in mice around E8.75-9 [2,23,44,45].

1.1.2.2 Morphogenesis and differentiation of the pancreatic lineages

Following endodermal patterning and specification of the pancreatic anlage, active proliferation of the pancreatic progenitors and cell shape changes drive the formation of two pancreatic buds [2,3,21]. During this stage, referred to as “primary transition” (E8.5-12.5), the buds appear as a pseudostratified epithelium, including a multi-layered core of unpolarized cells engulfed by a basement membrane [3]. Subsequently the two pancreatic buds elongate and ultimately fuse into a single organ by E12.5 as the rotation of the gut tube brings the ventral pancreatic bud into close proximity to its dorsal counterpart [2,3]. Concurrently, branching morphogenesis begins in tight temporal and spatial coordination with growth and differentiation of the pancreatic progenitor cells. Specifically, between E11.5 and E12.5 individual pancreatic epithelial cells acquire apico-basal polarity, undergo apical constriction and organize into rosettes around a central lumen [46–48]. By E15.5 the newly formed microlumens coalesce into a luminal plexus, which up to birth undergoes further remodelling into a highly branched ductal tree [3,23,49]. Concomitant, blood vessels and nerves penetrate the epithelium and intercalate between branches of the pancreatic ductal tree [50].

Along with branching events, the pancreatic progenitor cells differentiate stepwise into their respective lineages [46,51,52]. First, pancreatic progenitors segregate into tip and trunk domain at the onset of the secondary transition (E12.5) [3,53,54]. The tip cells, marked by the expression of *Ptf1a, Myelocytomatosis oncogene* (*c-Myc*) and *Carboxypetidase A1* (*Cpa1*), are highly proliferative and retain their ability to differentiate into all pancreatic cell types until E14.0 [2,53,55]. Later on, these multipotent tip cells become restricted to the acinar lineage, expressing *Ptf1a*, *Elastase* and finally *Amylase*. Acinar cell differentiation is mostly completed by E15.5 [23,45,46,53,56]. In contrast, the trunk domain consists of bipotent ductal/endocrine progenitors that express *NK6 homeobox 1* (*Nkx6.1*), *Nkx6-2*, *SRY-box 9* (*Sox9*), *HNF1 homeobox B* (*Hnf1b*), *One cut domain 1* (*Onecut1*), *Prospero homeobox 1* (*Prox1*) and *Hairy and enhancer of split* (*Hes1*) [3,52,57]. A small subset of trunk cells transiently expresses *Neurogenin 3* (*Ngn3*) and becomes committed to an endocrine cell fate. Instead, cells failing to turn on *Ngn3* undergo a ductal fate program [2,58,59]. Endocrine precursors further differentiate into the five distinct hormone producing cell types. This process is temporally controlled, where $Ngn3^+$ cells give first rise to α-cells, then β- and δ-cells, and finally PP-cells, though, the precise mechanism is unknown [60]. $Ngn3^+$ precursors exit from the cell cycle, delaminate from the trunk into the surrounding mesenchyme and cluster together to form pancreatic islets [23,56,61,62]. Notably, insulin and glucagon expressing cells appear already before the “second transition”, around E10.5 [60]. These cells are often bihormonal and considered as immature endocrine cells that do not contribute to mature islet cells. So far, their fate is not clear [23].

1.1.3 Intrinsic factors governing pancreas development

Pancreas development, from specification, over differentiation, to morphogenesis, is tightly controlled by a network of TFs with extensive cross-regulation between individual factors [2,3,23,46]. Through genetic studies and lineage-tracing experiments in animal models, key intrinsic factors orchestrating pancreas development have been elucidated [53,56,63]. These cell-intrinsic factors include TFs, microRNAs and epigenetic regulators. Furthermore, genetic analyses of patients suffering from rare monogenic forms of diabetes have confirmed the importance of certain intrinsic factors for pancreas development and will be discussed in more detail in section 1.2.2 [64–68].

1.1.3.1 Specification of the pancreas progenitors: Pdx1, Ptf1a, and Sox9

Expression of the pancreas master regulator *Pdx1* marks the region of the endoderm committed to pancreatic fate. *Pdx1* is a TF belonging to the ParaHox family initially cloned in *Xenopus* [69]. Morpholino knockdown of *Pdx1* in *Xenopus* resulted in the complete absence of acinar cells, while effects on β-cells were not reported [70,71]. Furthermore, overexpression of an activated form of *Pdx1* in *Xenopus* demonstrated that *Pdx1* promotes the conversion of liver to pancreas [71,72]. In mice, expression of *Pdx1* is induced at E8.75 in the two prospective pancreatic domains of the foregut. Later on, at E9.5, *Pdx1* expression expands more broadly and can be detected in the duodenum, bile duct and posterior part of the stomach [23]. Over the course of differentiation, *Pdx1* expression becomes mainly confined to β-cells [23]. Numerous genetic mouse models have underscored the role of *Pdx1* during pancreas development and glucose homeostasis [3]. *Pdx1* null mice develop pancreatic aplasia as the pancreatic epithelium loses its competence to respond to surrounding mesenchymal signals [73]. Indeed, pancreatic buds and few insulin- and glucagon-positive cells are formed, thus indicating that *Pdx1* is not required for pancreatic specification but the buds do not expand [73]. Conditional ablation of *Pdx1* in adult β-cells impairs β-cell function and identity, resulting in overt diabetes [74–76]. Consistently, homozygous mutations in the human *PDX1* gene lead to pancreatic agenesis, whereas heterozygous mutations cause MODY4 [67,77,78]. Therefore, *Pdx1* is highly conserved across vertebrate in terms of sequence, expression and function [79,80].

Shortly after the onset of *Pdx1* expression, *Ptf1a* starts being expressed in pancreatic progenitors [23]. PTF1a, along with a common E-protein partner and the bHLH factor RBPJ, forms the trimeric TF complex PTF1-J [81]. The expression of *Ptf1a* creates a clear tissue boundary in the foregut endoderm between the prospective pancreatic and the duodenal territory [81–84]. By E12.5, the expression of *Ptf1a* becomes restricted to the pancreatic tip cells, which later give rise to the acinar cells [23]. *Ptf1a* null mice display ventral pancreatic agenesis with a total lack of acinar cells, while few endocrine cells are still found. Moreover,

ventral pancreatic cells acquire an intestinal fate, whereas a dorsal rudiment is formed. In *Xenopus* endodermal overexpression of *Pdx1* and *Ptf1a* in a developmental context can convert prospective liver, anterior stomach and duodenal progenitors into *bona fide* pancreatic tissue [70,71]. Hence, *Ptf1a* is essential during pancreas development to establish pancreatic identity and tissue boundaries, as well as to promote an exocrine cell fate. Various lineage tracing experiments showed that the pancreatic epithelium differentiates after E12.5 into at least two progenitor regions–the PTF1A$^+$/CPA$^+$ tip and the SOX9$^+$/HNF1b$^+$/NKX6.1$^+$ trunk–that later differentiate into acinar or ductal and endocrine cells, respectively [3,53,85].

Maintenance of pancreatic identity is further reinforced by *Sox9*. The HMG box TF, SOX9, is expressed shortly after *Pdx1* in the pancreatic progenitor pool [86]. During the second transition, *Sox9* is gradually confined to the ductal cells of the trunk domain. In mice, pancreas-specific ablation of *Sox9* causes pancreatic hypoplasia likely due to depletion of the progenitor cell pool [86]. Moreover, during early development, SOX9, FGF2, and FGF10 form a feed-forward loop ensuring progenitor proliferation [87]. Additionally, *Sox9* is fundamental for establishing pancreatic cell identity since *Sox9*-deficient pancreata downregulate *Pdx1* and activate a hepatic transcription program [86]. *Sox9* also maintains pancreatic progenitor in an undifferentiated state, by controlling the Notch-effector *Hes1* [86,88]. During endocrine differentiation, *Sox9* is required to maintain *Pdx1* and *Ngn3* expression [89,90]. Heterozygous loss of *Sox9* impairs generation of NGN3$^+$ endocrine progenitors, which arise from the SOX9$^+$ progenitor pool in the trunk region [89]. Once *Ngn3* expression is induced, *Sox9* downregulation is required to specify endocrine *versus* ductal cell fate. Thus, *Ngn3* and *Sox9* are linked via a negative feedback loop, where *Sox9* positively controls *Ngn3* expression, and *Ngn3* negatively regulates the expression of *Sox9*. As a result, *Sox9* mutant pancreata also exhibit defects in duct differentiation that manifest, for example, as polycystic ducts that lack primary cilia [88,91]. Taking together, SOX9 is a pivotal TF in pancreas development and its temporal regulation governs cell lineage divergence within the pancreatic lineage decision (Fig. 2) [3,92].

In addition to these TFs described above, pancreatic progenitors express a wide range of other factors, which further contribute to the maintenance and expansion of the multipotent progenitor pool, including *Prox1*, *FoxA2*, *Onecut-1*, *Hes1*, *Nkx.2.2*, *Nkx6.1*, *Gata4* and *Gata6* [3,23,46]. Each of these genes is important during early pancreatic development and together they form a complex, interconnected gene regulatory network. Interestingly, these TFs also play a role later during pancreatic differentiation and maintenance of cell identity. Thus, the same TF can exert multiple functions depending on temporal and cellular context. Consequently, mice lacking any one of these factors display varying degrees of pancreas hypoplasia, agenesis or endocrine defects.

1.1.3.2 Endocrine differentiation: Ngn3, Isl1, and Neurod1

The SOX9^{+} bipotent trunk domain is poised to differentiate either into endocrine or duct cells depending on the induction of *Ngn3*, a pro-endocrine TF. In mice, *Ngn3* is expressed starting from E9.0 in scattered progenitor cells [23]. Moreover, several studies showed that the expression of *Ngn3* in mice is biphasic, correlating with the first and second transition, respectively [93,94]. Additionally, there is evidence that the dose of *Ngn3* expression plays a role during endocrine fate acquisition, as such, an increase in *Ngn3* levels (i.e. in NGN3^{high} cells) triggers endocrine commitment, cell cycle exit and delamination from the epithelium. In contrast, NGN3^{low} cells differentiate into acinar and ductal cells [61,94]. Johansson et al. further demonstrated the existence of a competence window for the generation of different endocrine subpopulations in the mouse; *Ngn3* induction at early stages of pancreas development generates foremost α-cells, whereas induction at E11.5 or E14.5 favours the formation of β-/PP-cells or δ-cells, respectively. However, the underlying mechanisms of this spatiotemporal *Ngn3* induction and regulation are largely unknown. The Notch effector HES1 has been shown to repress *Ngn3* transcription and promote NGN3 protein destabilization [95,96].

Ngn3 activates a set of TFs, among them the LIM homeobox TF Islet1 (ISL1), Neurogenic differentiation 1 (NEUROD1) and Insulinoma-associated 1 (INSM1), which are crucial for further endocrine differentiation [58,62,97,98]. Lineage tracing has illustrated that all islet endocrine cell types derive from NGN3^{+} cells [99]. Moreover, *Ngn3*-deficient mice completely lack endocrine cells and die from diabetes shortly after birth [58,100]. Instead, ectopic expression of *Ngn3* or its downstream target *NeuroD1* in the pancreas is sufficient to induce differentiation of endocrine cells in the mouse [27,101,102]. *NeuroD1* expression starts in the early pancreatic domain from E9.5 in the mouse and becomes later confined to the β-cells [103,104]. Mice deficient for *NeuroD1* display arrested endocrine expansion, increased endocrine cell apoptosis, accompanied by an overall abnormal islet architecture. Hence, NeuroD1 null mice develop diabetes and succumb perinatally [103]. The other NGN3 downstream target, *Isl1*, is expressed at E9.0 in the dorsal pancreatic epithelium, as well as, in the surrounding mesenchyme [105]. Its function is exemplified in mice full knockout embryos, which completely lack dorsal pancreatic mesenchyme and endocrine islet cells [105]. This is attributed to a decline in adult pancreatic islet cells proliferation and progressive cell apoptosis. In addition, the mature β-cell marker *MafA* was shown to be a target of *Isl1* [106]. In summary, *Ngn3* activates the expression of crucial TFs in endocrine precursors to regulate delamination, proliferation and final endocrine cell fate allocation.

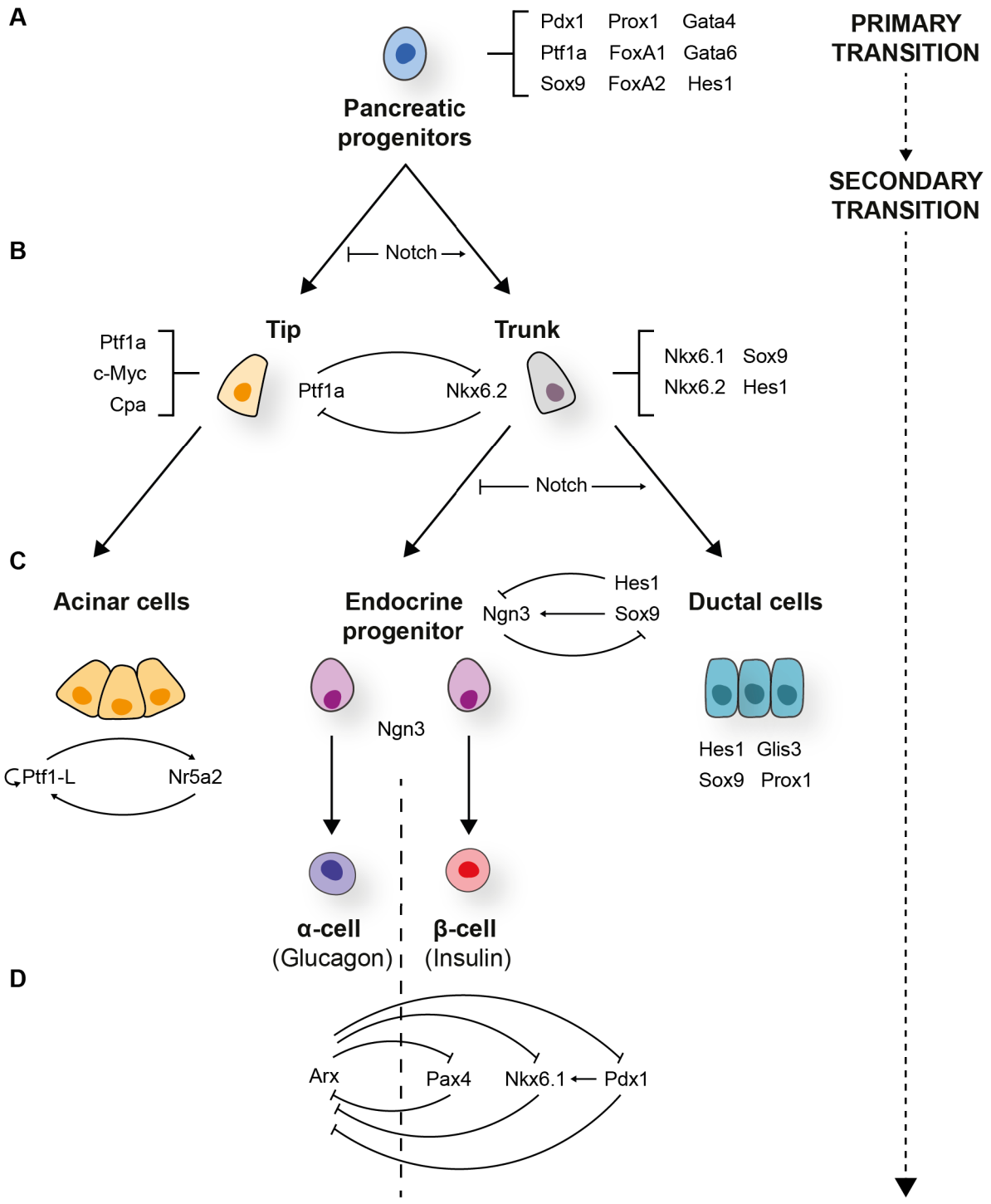

Fig. 2: Intrinsic transcriptional cascade regulating pancreas lineage decision. Schematic representation of pancreatic progenitor cell differentiation along the acinar, endocrine and ductal lineages. (**A**) During the primary transition, a set of TFs, including Pdx1, Ptf1a, and Sox9, mediates expansion of multipotent pancreatic progenitors and maintains their identity. (**B**) At the onset of the secondary transition, pancreatic progenitors segregate into the tip and trunk domains. Notch signalling and cross-repression between *Ptf1a* and *Nkx6.1* is important to establish a tip and trunk cell identity. (**C**) Tip cells further differentiate into acinar cells through the action of the PTF1-L complex. The trunks cells are bipotential for the ductal and endocrine cell fate. The latter is marked by the expression of *Ngn3*. (**D**) Endocrine progenitors further differentiate into hormone-producing cells. Mutual repression between the α-cell specific transcription factor *Arx* and the β-cell specific transcription factors *Pax4*, *Nkx6.1*, and *Pdx1* are required for the establishment and maintenance of α- and β-cell identity, respectively. Adapted from Shih et al., 2013.

1.1.3.3 Acquisition of β-cell identity: Nkx6.1 and Nkx2.2

Both NK-homeodomain genes, *Nkx6.1* and *Nkx2.2*, are expressed in pancreatic progenitors, but are not crucial for early pancreas development, playing rather an important role later in β-cell lineage differentiation [23]. Accordingly, their expression pattern changes and is confined to the trunk cells after the secondary transition [46,55]. While *Nkx2.2* is expressed in α-, β- and PP-cells of the mature islets, *Nkx6.1* is expressed exclusively in β-cells [23,107,108]. *Nkx6.1* and *Nkx2.2* null mice display normal pancreas specification, but β-cell differentiation is impaired. In particular, *Nkx6.1* null mice display loss of β-cell precursors, while the other endocrine cell types develop normally. Contrary, in mice lacking *Nkx2.2*, β-cells are almost completely absent and the number of α- and PP-cells are severely diminished, whereas the number of ε-cells is markedly increased [108]. Moreover, *Nkx6.1* expression is lost in endocrine cells, indicating that *Nkx6.1* acts downstream of *Nkx2.*2 in the transcriptional cascade promoting β-cell differentiation [107,108]. Notably, NKX2.2 has been shown to form a complex with DNMT3a, GRG3/TLE3 and the histone deacetylase HDAC1 to repress α-cell identity in β-cells [109]. Lineage decision in favour for β-cell at the expense of α-cell is also mediated by the direct repression of the α-cell-specific TF Aristaless-related homeobox (ARX) in β-cell precursor through NKX6.1. Consistently, ectopic overexpression of *Nkx6.1* converts endocrine precursor towards the β-cell lineage [109].

1.1.3.4 Regulation of α-versus β-cell fate decision: Pax4, Pax6, Arx, Mafa, and Mafb

During fate decision between β- and α-cells, two TFs, namely PAX4 and ARX, play an important role. Both are expressed in the developing pancreas starting from E9.5 and, progressively, become restricted to specific endocrine cell types [110,111]. In particular, *Pax4* expression is limited to first and second wave β-cells but is down-regulated perinatally and not detectable in adult islets [111]. In mice, ablation of *Pax4* expression leads to loss of mature β- and somatostin-producing δ-cells, and a concomitant increase in the number of α-cells [111]. Conversely, ectopic expression of *Pax4* is sufficient to convert α-cells into β-cells *in vivo* [112]. In contrast, *Arx* expression becomes later restricted to α- and PP-cells. *Arx*-deficient mice display a complete opposite phenotype to *Pax4* knockout mice, namely an increase in β-cells at the expense of α-cells [113]. In line with this, *Arx* gain of function results in excess α-cells and reduced β-cells [113]. Thus, *Pax4* favours the β-cell fate choice, while *Arx* promotes a α-cell fate and represses a β-cell destiny. Mechanistically, this is established through a mutually antagonistic transcriptional network between PAX4 and ARX, which are both activated by NGN3 [114]. Specifically, PAX4 inhibits the expression of *Arx* and vice versa, thereby mediating proper endocrine fate allocation. In addition, the establishment of β-cell identity relies on reciprocal repression between *Arx* and *Nkx6.1* [115]. Interestingly, loss of *Pax4* and

Arx causes a massive expansion of δ-cells, indicating that repression of both TFs is required for induction of δ-cell fate [114].

Another Pax family TF required for proper endocrine commitment is Pax6 [110]. In fact, $Pax4^{-}$/$Pax6^{-}$ double mutants lack entirely endocrine cells [116]. *Pax6* is expressed as early as E9.0 in the developing pancreas and after the second transition becomes limited to endocrine cells. In adult islets, *Pax6* expression can be found in α-, β-, δ- and PP-cells [110]. Mouse genetic studies have underscored the requisite of *Pax6* for both endocrine cell fate decision and maintenance of endocrine cell identity. Respectively, *Pax6* knockout mice display abnormal islet organization with marked α-cell reduction relative to other endocrine cell types and thus die shortly after birth [110,116]. Conditional inactivation of *Pax6* in adult β-cells results in diabetic phenotype with hypoinsulinemia, coupled with an increase in the number of ghrelin-positive cells, a gut hormone normally expressed only transiently in the fetal pancreas [117]. Moreover, *Pax6* deletion in adult β-cells showed downregulation of mature β-cell signature genes, like *MafA* and *Ins*, and derepression of alternative islet cell genes and hormones [118]. Therefore, *Pax6* can function mechanistically as an activator of β-cell genes (e.g. *Pdx1*, *MafA* and *Nkx6.1*) and repressor of β-cell “disallowed” genes (e.g. *grehlin*, *somatostatin*) in the pancreas.

Following initial lineage segregation into β- or α-cells, respectively, subsequent functional maturation and maintenance of cell identity is orchestrated by two bZIP factors, namely MAFA and MAFB. *MafB*-null embryos show a reduced number of $insulin^{+}$ and $glucagon^{+}$ cells. Moreover, the expression of crucial β-cell genes, involved in glucose sensing and insulin secretion, is reduced in residual endocrine cells [119]. In contrast, *MafA* expression is directly restricted to insulin-secreting β-cells and serves as a marker for terminally differentiated β-cells [120]. For β-cell maturation, a switch from *MafB* to *MafA* expression is pivotal [121]. MafA is under the regulation of β-cell specific TFs, such as NEUROD1, NKX6.1 and PAX6, and together with other factors it controls the transcription of Insulin. Consequently, the absence of *MafA* in mice leads to diabetes, characterized by glucose intolerance, altered glucose-stimulated insulin secretion and perturbed islets architecture [122].

In summary, multiple TFs play an important role during distinct stages of pancreas development and adult organ function. With the knowledge gained from analysing pancreas development, researchers are exploring novel therapeutic avenues for the treatment of diabetic patients by restoring functional β-cell mass. Significant progress has been made in understanding several intrinsic factors, yet how these are orchestrated to activate and regulate individual fate decisions is still poorly understood. Importantly, recent studies have underscored that additional factors, like epigenetic modulators and mesenchymal signals, influence pancreatic fate decisions. These add another level of complexity and may contribute to further fine tune cellular responses to TFs.

1.1.4 Epigenetic regulators in pancreas development and β-cell function

Pancreas development is controlled by a complex interaction of signalling pathways and TF networks that regulate pancreatic specification and differentiation of exocrine and endocrine cells. Epigenetics adds an additional layer of gene regulation where the DNA and its associated proteins and ribonucleoproteins are modified without altering the DNA sequence itself [123]. These mechanisms can be broadly classified into: (a) posttranslational histone modifications (PTMs) and chromatin remodelling, (b) DNA methylation, and (c) non-coding RNA interactions [124]. Moreover, the interplay of these epigenetic modifiers in intra- and internucleosomal interactions over short (cis) and long (trans) distances leads to changes in the chromatin state [125]. The latter determines the accessibility of regulatory proteins to DNA and shapes the three-dimensional organization of the genome. As a consequence, the chromatin state and epigenetic mechanisms affect various processes, including DNA recombination, replication, mitotic condensation and transcriptional regulation [124,126]. Therefore, altered epigenetic processes might contribute to pancreatic diseases, like diabetes and pancreatic cancer [127–129].

1.1.4 .1 Post-translational modifications of histones

The genomic material in eukaryotic cells is packed at multiple levels to build up the chromatin. The repeating unit of chromatin is the nucleosome, which is formed by linear DNA (~145 to 147 bp long) wrapped around a histone octamer and stabilized by the linker histone H1 [130,131]. Each histone octamer encompasses two copies of the histone H2A, H2B, H3 and H4. Nucleosome complexes occur on average every 200 bp and undergo further chromatin compaction to form higher order structures [130].

The N-terminal tails of the core histones protrude from the nucleosome and are subjected to various covalent modifications, which alter chromatin packaging and recruit transcriptional regulators to modify gene expression in a multitude of ways. Among the different histone modifications are acetylation, methylation, and phosphorylation [132]. These PTMs are introduced by a variety of histone modifying enzymes that can add or remove covalent modifications and are called “writers” or “erasers”, respectively [133]. Their actions to govern DNA transcription are mediated by effector proteins or “readers”. Readers contain protein domains that allow their recruitment to chromatin. For instance, the bromodomain recognizes acetyllysine and the plant homeodomain (PHD) finger recognizes acetyllysine, methyllysine, and even unmodified lysine [133].

Histone acetylation is highly dynamic and regulated by the opposing action of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs transfer an acetyl group from acetyl-CoA to the ε-amino of histone lysine residues resulting in acetylated lysine and CoA [134]. Thereby the positive charge of lysine is neutralized and the interactions between

histones and DNA are destabilized. Thus, HATs facilitate gene activation, as the chromatin becomes more accessible to interacting proteins. The reverse reaction is catalysed by HDACs, which induce deacetylation, chromatin condensation and transcriptional repression [132].

Like histone acetylation, phosphorylation of histones is highly dynamic. It occurs at serine, threonine and tyrosine residues and is controlled by kinases and phosphatases that add and remove the modifications, respectively. Histone kinases transfer a phosphate group from adenosine triphosphate (ATP) to the hydroxyl group of the target amino-acid side chain [132]. This adds a significant negative charge to the histone altering the chromatin structure [132,135]. While numerous phosphorylation sites on histones have been described, much less is known about their biological functions.

Besides acetylation, histone lysine and arginine residues are subject to methylation. Lysine residues can be mono-, di- and tri-methylated by lysine methyltransferases (KMT), whereas arginine residues are only subject to mono- and di-methylation by arginine methyltransferases (PRMTs) [132]. Contrary to histone acetylation, methylation does not alter the charge of the histone protein. Instead, the regulation of transcriptional activity relies on the extent and localization of methylation. In detail, methylation at histone H3 lysine 4 (H3K4; H3K4me2, H3K4me3) and H3K36 (H3K36me3) are generally linked to gene activation, whereas those at H3K9 (H3K9me2, H3K9me3) and H3K27 (H3K27me2, H3K27me3) are usually associated with gene silencing [132,136]. Epigenetic methyl-marks can be “erased” by lysine or arginine demethylases. The lysine-specific demethylase 1 (LSD1) removes, for example, methyl groups from H3K4me1/2 by utilizing flavin adenine dinucleotide (FAD) as a co-factor [137]. Interestingly, it was shown that the TF TBX3 associates with the histone H3K27 demethylase JMJD3 at the enhancer region of the endodermal regulator EOMES to drive the differentiation of human and mice PSCs towards DE lineage [138]. Moreover, the histone methyltransferase EZH2 and SETD7 have been reported to play a role in modulating the cell fate decision between liver and pancreas and establishing pancreatic cell identity, respectively [139].

Over the years, several histone modifications have been identified. Additional ones are ubiquitination and sumoylation of lysines, arginine deamination and proline isomerization [124,132]. Yet, their function remains to be elucidated. However, much effort has been devoted to understand how histone modification regulates development and disease. Techniques, like Chip-seq and ATAC-seq, allow nowadays genome-wide mapping of the binding of chromatin regulators and epigenetic modifications, as well as, profiling of chromatin accessibility [140–142].

1.1.4 .2 The role of HDACs during endocrine cell development

HDACs are well known epigenetic modulators and play diverse roles in development and tissue homeostasis. Mammalian HDACs are divided into four distinct classes based on their phylogenetic conservation to yeast prototypes, catalytic sites and cofactor dependency. Class I and II HDACs are zinc-dependent enzymes [143]. Class I HDACs (related to yeast Rpd3) comprise HDAC-1, -2, -3 and -8. Instead HDAC-4, -5, -7 and -9 belong to the sub-class IIa (related to yeast Hda1), while HDAC-6 and -10 are members of the sub-class IIb [144]. Class III, includes of seven sirtuins (SIRT1-7), require nicotinamide adenine dinucleotide (NAD^+) as a co-factor and class IV, represented by HDAC11, has a catalytic region which is similar to both class I and II HDACs [145]. Class I HDACs are predominantly located in the nucleus and ubiquitously expressed. In contrast, class II HDACs can shuttle between the nucleus and the cytoplasm and display a tissue-specific expression pattern [145,146].

Dynamic changes in the acetylation of histones are also important during differentiation of pancreatic endocrine cell types. Key evidence comes from the use of specific HDAC inhibitors and gene ablation models in mice. Lenoir et al. illustrated that the class IIa HDACs members, HDAC-4, -5, and -9, are specifically expressed in β- and/or δ-cells during mouse pancreas development at E15.5 and in adulthood. However, expression of these HDACs is absent in glucagon-producing cells and acinar cells. In more detail, HDAC-4 is highly enriched in δ-cells, HDAC-5 in β- and δ-cells, and HDAC-9 in β-cells [147]. The fourth member of the class IIa HDACs, HDAC7 is not expressed in endocrine cells but present in vascular endothelial cells [147]. Moreover, lentiviral-mediated overexpression of *Hdac4* in rat pancreatic spheres (E13.5) involved a reduction in the expression level of *Insulin*, *Somatostatin* and genes required for β-cell differentiation and function (e.g. *Pdx1*, *MafA*, *NeuroD1*). Consistent with these results, treatment of pancreatic spheres with a selective class II HDAC inhibitor led to an induction of *Pax4*, which is involved in β- versus δ-cell fate. This was further associated with an increase in both *Insulin* and *Somatostatin* expression [147]. Another study in mouse pancreatic β-cell lines indicated that HDAC4 regulates insulin secretion, expression of β-cell markers and deacetylation of FoxO1. The TF FoxO1 induces the expression of *NeuroD1* and *MafA*, and is therefore crucial for β-cell function [148]. Additionally, acetylation of FoxO1 inhibits the interaction with the glucose-6-phosphotase (G6P) promoter by disrupting the FOXO1-DNA complex [148,149]. Further investigations are necessary to define the underlying mechanism by which HDACs modulate pancreas development and endocrine cell differentiation.

HDAC inhibitors are powerful epigenetic regulators and promising tools to modulate cell differentiation in a variety of lineages. Indeed, small molecules that target both class I and II HDACs have been identified to drive differentiation of mouse embryonic stem cells (ESCs) into DE. For example, the putative HDAC inhibitors IDE1 and IDE2 induce the expression of

the endodermal marker *Sox17* [150]. The class I HDAC inhibitors sodium butyrate and valproic acid have been used in combination with activin A to prime human ESCs towards DE [151,152]. Another HDAC inhibitor (5'-Azadeoxycytidine) has been described to promote endocrine lineage development by stimulating the expression of *Ngn3* [153]. Moreover, HDAC inhibitors have been used to increase reprogramming efficiency of somatic cells into iPSCs. For example the HDAC inhibitor valproic acid enables efficient induction of pluripotency with only two Yamanaka factors, *Oct4* and *Sox2*, instead of four [154].

To summarize, HDACs play a crucial role in guiding lineage commitment and regulating cell differentiation. The above-described findings emphasize the potential application of HDAC inhibitors in therapeutic cell reprogramming and directed differentiation of pluripotent stem cells (PSCs) into pancreatic cells. Notably, HDAC inhibitors are currently in preclinical trial for T2D [155]. Finally, the putative benefits of HDAC inhibitors as treatment for diabetes and the role of HDACs during endocrine development merits further investigations.

1.1.5 Extrinsic factors involved in pancreas development

The developing pancreas is exposed to various extrinsic factors, such as secreted growth factors, morphogens, and cell-surface ligands, derived from the surrounding tissues. Previous studies showed that signalling molecules released by the notochord, dorsal aorta and the surrounding mesenchyme, as well as epithelial-mesenchymal cell interactions, are crucial determinants of pancreatic fate decisions, proliferation, branching and differentiation. All major signalling pathways, such as BMP/TGF-β, FGF, Sonic hedgehog (SHH), WNT, Retinoic acid (RA) and Notch have been described to play a role at distinct steps of pancreatic development; a selection of them is discussed in more detail below [2,3,5,43].

1.1.5.1 TGFβ / BMP signalling pathway

Multiple components of the TGFβ signalling pathways are expressed in the embryonic pancreas epithelium and mesenchyme. These include the TGFβ ligands activin, BMP and TGFβ1, which bind to heteromeric receptor complexes composed of membrane-associated type I and type II receptor kinases [156,157]. Ligand activation of these receptors triggers phosphorylation of intracellular proteins named receptor-regulated Smads (R-Smads), which form complexes with Smad4 [158]. The activated Smad complex then translocates to the nucleus, where they control the transcription of target genes [159]. Distinct R-Smads mediate the BMP or TGFβ and activin signalling, respectively. For instance, R-Smads1, 5 and 8 transduce BMP signalling, while R-Smads2 and 3 mediate TGF-β and activin signalling [158]. Moreover, inhibitory Smads, like Smad6 and Smad7, block R-Smad phosphorylation and thus

TGFβ/BMP signalling [160]. In addition, ligand antagonists, including follastatin, noggin, and gremlin are expressed in the pancreas [161].

Activins and their receptors are broadly expressed in the early foregut endoderm and pancreatic rudiments [162,163]. Specifically, activin A and B localise to the developing endocrine cells, being highly expressed in the α-cells [162]. At this early stage of development, TGFβ and activin signalling is necessary for pancreatic morphogenesis and endocrine differentiation. Consistently, mice harbouring null mutations in the type II activin receptors ActRIIA and ActRIIB exhibit impaired glucose tolerance, hypoinsulinemia and hypoplastic pancreas islets [164]. Smad2, the signalling transducer of both ActRIIA and ActRIIB, prevents β-cell failure in adult mice since $Smad2^{+/-}$ heterozygous mice exhibit β-cell hypoplasia, hypoinsulinemia and glucose intolerance [165]. It is important to note that complete *Smad2* knockout mice fail to form endoderm [165,166]. Furthermore, *in vitro* exposure of embryonic mouse pancreas to activin disrupts branching morphogenesis and promotes the development of insulin-expressing endocrine cells [167,168]. Instead, treatment with follistatin, an activin inhibitor, resulted in enhanced exocrine differentiation at the expense of endocrine cell formation. Consistently, follistatin like-3 knockout mice have enlarged islets resulting from β-cell hyperplasia, increased glucose tolerance and slight hyperinsulinemia [158]. Moreover, activin A is capable of inducing differentiation of human fetal pancreatic endocrine cells into β-cells by potentially stimulating the expression of *Pax4* and *Insulin* [167].

BMP molecules are expressed in the pancreas and BMP signalling has been implied to play a role during both pancreatic epithelium and mesenchyme development [169,170]. However, results from different animal models have highlighted the dynamic requirements for BMP signalling during pancreatic development. For instance, BMP4 overexpression under the control of the PDX1 promoter in transgenic mice does not affect pancreas development or pancreatic cell differentiation but these mice display an increased glucose tolerance and insulin secretion [171]. Moreover, systemic administration of BMP4 to adult mice stimulates insulin secretion, while BMP7 was shown to promote the conversion of pancreatic exocrine tissue into functional β-cells [171,172]. Consistently, conditional deletion of the BMP receptor 1A (Bmpr1a) in adult β-cells leads to severe glucose intolerance without affecting pancreas development [171].

1.1.5.2 Hedgehog (HH) signalling pathway

In mammals, three hedgehog genes, Sonic (*Shh*), Indian (*Ihh*) and Desert hedgehog (*Dhh*), have been isolated that are expressed during embryogenesis and pivotal for the development of numerous organs, including brain, gut and pancreas [173–179]. Hedgehog genes code for secreted proteins that bind to their membrane bound receptors, called Patched (Ptch). Two homologous receptors exist in mammals, referred to as Ptch1 and Ptch2 [180]. In

the absence of Shh, Ptch inhibits the activity of Smo. The binding of Shh to Ptch results in the release of Ptch inhibition of Smo and, subsequently, in the activation of the downstream GLI TFs, namely GLI1, GLI2 and GLI3. Activated GLI TFs shuttle into the nucleus where they act as transcriptional repressor or activator of numerous target genes, including components of the pathway itself, such as *Ptch* [177,181,182].

During pancreas development, HH signalling is tightly regulated. Initially, *Shh* repression is required for the specification of the pancreatic anlage in the foregut endoderm [173]. Seminal studies in chick have demonstrated that the notochord, a mesodermal structure, is necessary for the induction of the pancreas differentiation program [183]. In this context, the notochord produces factors that repress the expression of *Shh* locally in the gut endoderm, fated to form the dorsal pancreatic bud. Subsequently, the restrictive effect on *Shh* expression in the dorsal endoderm is relieved when the notochord and pancreatic epithelium become separated by the fusion of the dorsal aorta [183–185]. Nevertheless, SHH remains excluded from the dorsal and ventral pancreatic buds [185]. Consistently, ectopic expression of *Shh* in the pancreatic endoderm under control of the *Pdx1* promoter results in significant loss of exocrine and endocrine tissue along with a transformation of pancreatic mesenchyme into duodenal mesoderm [186]. Moreover, Activin signalling inhibits Shh activity, with mutations in type II activin receptors in mice increasing Shh expression and impairing induction of pancreatic marker [164]. Like in the dorsal bud, the HH pathway needs to be repressed during ventral pancreas fate specification. However, this occurs via a notochord-independent mechanism since it derives from the ventral endoderm, which has no contact with the notochord [173]. Recent observations have proposed a repressive interaction between SHH and the Gata4/6 TFs [187].

Subsequently in development, altered HH signalling affects the development of endocrine and exocrine cells and diminishes β-cell function and mass. For the maintenance of endocrine function, active HH signalling is essential. Ectopic expression of SHH in cultured β-cell lines has beneficial effects on insulin production and secretion as well as on survival [185,188]. Moreover, these effects are in part mediated through the induction of the *Pdx1* promoter, a gene known to stimulate the expression of insulin [188]. Conflicting evidence also suggests that high HH levels in insulin-producing cells impairs β-cell function by interfering with the mature β-cell differentiation state. Landsman and colleagues demonstrated that elevated HH signalling in adult β-cells results in glucose intolerance in transgenic mice. Increased HH signalling further correlated with increased expression of *Sox9* and *Hes1*, both direct HH targets and normally only expressed in progenitors and not in adult β-cells [189]. Efforts have also been made to distinguish between HH signalling contributions to pancreas epithelium and mesenchyme. Recently, mesenchymal loss of HH regulators, Suppressor of Fused (*Sufu*) and Speckle-type POZ protein (*Spop*), was shown to impair progenitor growth

and β-cell genesis [190]. SUFU sequesters GLI TFs in the cytoplasm, while SPOP targets them for proteasomal degradation. Furthermore, the described pancreatic defects occur through GLI2-mediated HH signalling and overexpression of *Wnt* ligands in mice [190]. Thus, a precise spatiotemporal HH regulation appears to be important during pancreas development and β-cell function [191].

1.1.5.3 Wingless/integrated (Wnt) signalling pathway

The Wnt/β-catenin signalling pathway plays a role at numerous stages of pancreatic development, including specification, proliferation and differentiation. Wnt signalling pathway consists of two major branches: the canonical pathway (Wnt/β-catenin) and a non-canonical pathway, which is subdivided into the Wnt/Ca^{2+} and planar cell polarity (PCP) pathways [192]. With regard to canonical Wnt signalling, when a WNT ligand is not bound to its receptors, a so called “destruction complex” is constitutively active, which phosphorylates β-catenin in a glycogen synthase kinase 3β-dependent manner (GSK3β) resulting in ubiquitination and proteosomal degradation of β-catenin [193]. The destruction complex itself is composed of axin, adenomatous polyposis coli (APC), GSK3β, casein kinase-1 (CK-1) and β-transducin repeat containing protein (β-TrCP). Upon binding of Wnt ligands to the frizzled receptors (Fzd) and its co-receptor lipoprotein receptor-related protein (LRP) 5/6, dishevelled (Dsh) is recruited and the “destruction complex” disrupted, allowing the translocation of β-catenin to the nucleus [192–194]. Here, β-catenin complexes with the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) TF families to regulate the expression of target genes. Compared to the canonical pathway, much less is known about the β-catenin-independent or non-canonical pathway [195]. In addition to the many branches of signalling that are triggered by Wnt ligands, the large number of ligands, receptors, co-receptors and antagonists make the pathway highly complex and versatile. For instance, the secreted inhibitors Dickkopf (DKK) and secreted-frizzled-related peptide (SFRP) are tightly regulated during embryogenesis and potentially serve to establish a Wnt /β-catenin gradient crucial for pattern formation [193,196].

An early role for Wnt signalling in pancreatic fate specification has been reported in *Xenopus* and mouse embryos. Notably, canonical Wnt signalling needs to be inhibited in the anterior endoderm of *Xenopus* embryos to maintain foregut identity and allow pancreas and liver development [41,197]. After the foregut territory is determined, non-canonical Wnt ligands, like Wnt5A, control the pancreas *versus* liver cell fate decision in *Xenopus* [198]. Likewise, treatment of mouse ESCs with Wnt5A promotes pancreatic fate by inducing the expression of *Pdx1* [198].

During pancreatic development, various Wnt ligands localize in the mouse pancreatic mesenchyme, whereas Fzd receptors and Wnt antagonists are expressed in both the epithelium and mesenchyme. Misexpression or loss of Wnt components results in several

pancreatic defects. For instance, mice overexpressing *Wnt1* and *Wnt5A* under the control of the *Pdx1* promoter display pancreatic agenesis and pancreatic hypoplasia, respectively, due to perturbed patterning of the foregut [196]. In line with this, expression of activated β-catenin in the early pancreas leads to dramatic pancreatic hypoplasia [199]. Overexpression of a dominant-negative form of mouse *Fz8* in pancreatic progenitors severely diminishes pancreatic growth and affected both exocrine and endocrine compartments in mice [200]. Nevertheless, transgenic mice remain normoglycemic and display normal glucose tolerance. Contrary, ablation of *Lrp5* and overexpression of the Wnt inhibitor *Axin1* in β-cells leads to impaired insulin secretion and β-cell proliferation, respectively [201]. Moreover, mice null for the core Wnt-PCP surface receptors *Celsr2* and *Celsr3* display a reduction in differentiated endocrine cells. More recently, the Wnt-PCP pathway has also been shown to be important for pancreatic β-cell maturation in mouse and human [202].

1.1.6 Human pancreas development

Over the past two decades, remarkable progress has been achieved in terms of understanding the mechanisms that govern pancreas development. However, our insight has been largely gained from comprehensive studies in animal models, like *Xenopus*, zebrafish, chicken and particularly mice. Based on the premise that cellular and molecular pathways are evolutionary conserved, these results have been transferred to human. Numerous studies have reinforced this concept and current protocols based on mouse developmental biology are used to generate human β-like cells *in vitro*. However, caution must be exercised, as differences between mice and human pancreas development clearly exist.

Studies of human pancreas development are generally hindered by the limited accessibility to early human fetal tissues [203]. Yet, several research groups have started investigating key processes of pancreas organogenesis in human tissues. This work, together with intensive studies on pancreatic cells obtained through *in vitro* differentiation and immortalized human β-cell lines, allowed to partially decipher the roadmap of human pancreatic lineage formation [18,20,203–206]. Although similarities between pancreas development in animal models and human exist, several reports have highlighted key discrepancies [207–209]. For instance, differences in early pancreatic development, endocrine cell ratio and islet structure between mice and human are present [8].

In humans, like in rodents, dorsal and ventral pancreatic buds emerge from the foregut endoderm, which eventually fuse together to form a single organ primordium at 6-7 weeks of gestation (G6-7w) [1]. However, only a single wave of endocrine cell formation occurs in the developing human pancreas [210]. Moreover, unlike rodents, the TF *NKX2.2* is not expressed in pancreatic progenitors before endocrine commitment [211]. As early as G7w, a tip and trunk

domain can be distinguished by the expression of GATA4. At this stage, trunk cells have less GATA4 and are positive for SOX9, NKX6.1, whereas the more peripheral tip cells are positive for SOX9, NKX6.1 and GATA4 [1,210,211]. Shortly afterwards, the CPA1/GATA4-positive tip cells no longer express *NKX6.1* and mostly lack *SOX9. NGN3* is also required for human endocrine cell differentiation as in the mouse. Comparable to rodents, *NGN3* is transiently expressed near the end of embryogenesis at G8w and initiates β-cell differentiation [1,210,211]. The first insulin-expressing fetal β-cells emerge around G7.5w, followed by the appearance of glucagon and somatostatin-expressing cells at G8w, and pancreatic polypeptide and ghrelin-expressing cells at G9w. By the last trimester, the majority of α-, β-, and δ-cells in the fetal human pancreas express a single hormone and reach approximately a 1:1:1 ratio, which is maintained throughout birth, while in mice, the β-cells are the most abundant endocrine cell type at birth [212,213]. Interestingly, the morphology of human islets changes during development. At 14 weeks post coitus (wpc), like in rodent islets, β-cells are found in the core and α-cells at the periphery, whereas later on at 21 wpc both cell types are intermingled within human islets [8]. This islet architecture might be crucial for human endocrine cells to reach their final mature functional state [214].

To generate in-depth understanding of pancreatic development and pathological mechanisms, major progress has been made towards modelling human pancreas organogenesis *ex vivo* through differentiation of human PSCs. Additionally, the establishment of new technologies, such as single-cell transcriptome profiling and single-cell resolution imaging might provide deeper insight into human islet-cell development, function and cellular heterogeneity in health and disease. Collectively, these investigations will be helpful to improve therapeutic approaches for the treatment of diabetes.

1.2 Diabetes Mellitus

Diabetes is a group of metabolic diseases characterized by hyperglycaemia and still a leading cause of mortality and morbidity worldwide [13,215,216]. The first description of diabetes can be dated back to more than 3500 years old manuscripts from ancient [217]. The term Diabetes mellitus - mellitus Greek for “like honey” - is coined to Thomas Willis, who in 1674 first distinguished diabetes from other causes of polyuria by the sweet taste of diabetic urine [217,218]. During the following centuries diabetes mellitus was traditionally thought of as a disease of the kidney [219]. However, the experimental induction of diabetes in pancreatectomized dogs in 1889 and ultimately the isolation of insulin 33 years later, clearly established diabetes as an endocrine disease caused by insulin deficiency [217,220–222]. This deficiency leads then to an increase of glucose in the patient’s blood and urine. Over the years, our understanding of the disease has advanced considerably. Nevertheless, diabetes has reached pandemic levels, affecting about 425 million people by 2018 and its prevalence is projected to increase by 48% within 2045 [223]. On top of this, it is estimated that globally half of the people living with diabetes are undiagnosed [223]. Currently, there is no cure for diabetes and therapeutic options are limited. Effective management of diabetes aims at sustaining lifelong glycaemic control [216]. Left untreated or managed poorly, the resulting chronic hyperglycaemia may lead to micro- and macrovascular complications, such as diabetic nephropathy, retinopathy and increase the risk of heart attacks and strokes [224]. Hence, diabetes represents an enormous health burden for the individual, as well as for the global economy, with direct annual cost of diabetes being estimated to be more than 827 billion USD [13,223]. A comprehensive understanding of the disease mechanisms is therefore necessary to reduce the incidence of diabetes, improve diagnosis/treatment, prevent complications and finally find a cure for diabetes.

1.2.1 Classification of Diabetes Mellitus

Diabetes is a very complex and heterogeneous disease with hyperglycaemia as the unifying clinical feature. Broadly, diabetes is classified into four etiological categories: Type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM) and other specific forms of diabetes [216,225]. In general, diabetes is diagnosed by blood or plasma glucose levels. For instance, a random plasma glucose level $\geq$ 200 mg/dl in a patient with classic symptoms of polyuria, polydipsia and polyphagia, is enough to make the diagnosis [225]. Proper classification is fundamental as it determines the strategy of treatment. However, in

some cases it is difficult to precisely define the type of diabetes, especially in children and young adolescents [66,216,226]. Therefore, other factors like severity of the disease, age of onset, family history of diabetes, obesity, ethnicity and presence of autoimmune antibodies are taken into consideration.

T2D is by far the most common form of diabetes, accounting for over 90% of all diabetes cases. It occurs more frequently in people older than 45 years but is increasingly seen in children and young adolescents as a consequence of the rising levels of obesity, lack of physical activity and fat-rich diets [10,216,227]. T2D is a result of relative insulin deficiency due to insulin resistance and β-cell failure. Once, the pancreatic β-cells cannot compensate for the increased insulin demand, hyperglycaemia and T2D develop. Up to date, many different mechanisms have been proposed to contribute to β-cell failure, including reduction in β-cell numbers, dysfunction of β-cells and loss of β-cell identity [228,229]. Despite differences in the underlying processes, the final outcome is the same: impaired insulin secretion. In contrast, insulin resistance refers to the metabolic actions of insulin, specifically the decrease of insulin-stimulated uptake of glucose into target tissues, like liver, muscle and adipose [230,231]. Furthermore, inappropriate insulin signalling in the liver fails to suppress hepatic glucose production and promotes lipid synthesis contributing to the progression of T2D [232]. As the severity of relative insulin deficiency can vary from patient to patient, so do treatment recommendations. In some individuals with T2D, glycaemic control can be achieved with lifestyle changes (e.g. diet, physical activity, weight loss) and/or oral glucose-lowering drugs (e.g. sulfonylureas, thiazolidinedione). For other individuals this is not sufficient, and they require exogenous insulin [10,216].

T1D accounts for 5% to 10% of all diabetes and mainly occurs in children and young adolescents [233,234]. In contrast to T2D, it is caused by an autoimmune destruction of the insulin-secreting β-cells, mainly through T-cell mediated inflammatory response, leading to an absolute insulin-deficiency [216,235]. Thus, patients depend on insulin administered by either multiple daily injections or insulin pump [236]. The presence of autoantibodies against pancreatic islet cells is the hallmark of T1D and identified in 90% of the cases at the time of diagnosis. These autoantibodies include islet cell autoantibodies, and autoantibodies against insulin (IAA), glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase (IA2 and IA2β) and zinc transporter protein (ZnT8A) [234]. Like T2D, T1D is polygenic in nature and arises from the interplay of multiple genetic, epigenetic and environmental factors.

Women who develop diabetes during their pregnancy are classified has having GDM. GDM often occurs in the third trimester of pregnancy and patients have an increased risk of developing T2D later in life [225].

Finally, the class of other specific forms of diabetes encompasses a variety of rare and diverse types of diabetes caused by genetic defects, diseases of the exocrine pancreas,

endocrinopathies, infections, drugs and hormones [225]. Monogenic forms of diabetes, arising from a single mutation in one gene, fall also into this group. They only account for approximately 1% to 2% of diabetes in children and adolescents but provided considerable insight into molecular pathways relevant to β-cell development and physiology [65,66]. Monogenic diabetes is subclassified into neonatal diabetes (NDM), maturity-onset diabetes of the young (MODY), mitochondrial diabetes and monogenic diabetes syndromes, like the Wolfram syndrome, which are characterized by glucose intolerance together with extra-pancreatic features [66]. Historically, NDM and MODY were distinguished based on the age at diabetes onset. For example, NDM occurs within 6 months of infancy, whereas MODY is typically diagnosed before the age of 25 years. Further, NDM can be transient or persist throughout life, named permanent NDM. To date, more than 30 genes have been linked to monogenic diabetes and the list continues to be expanded thanks to advances in genomic research [66].

Despite extensive research, our understanding of the underlying pathogenesis of diabetes remains elusive. Diabetes in all types has a strong genetic component and genome-wide association studies (GWAS) have helped discovering novel candidate genes and important genetic risk loci that are linked to the pathology of diabetes.

1.2.2 The Genetics of Diabetes

Genetic factors play an important role in susceptibility to common human diseases, like stroke and diabetes. The majority of common diseases are 30% to 60% heritable while lifestyle and other environmental factors also contribute to disease development. Over the last decade, the development of novel genomic technologies and data analysis tools has remarkably improved our ability to systematically identify new risk loci and candidate causal genes for diabetes. From a genetic point of view diabetes can be divided into polygenic (T1D and T2D) and monogenic forms. Nowadays, more than 100 susceptibility loci have been associated with diabetes through genetic linkage-studies, candidate-gene association studies but mainly through GWAS [237–239]. The main findings will be discussed in the subsequent sections.

1.2.2.1 The genetics of polygenic diabetes

The most common forms of diabetes, T1D and T2D, are polygenic in origin [80]. Genetic studies using linkage analysis and candidate gene approaches have substantially highlighted their strong genetic component. After the human genome was sequenced, GWAS offers now a more unbiased way to identify associations between genetic variants, known as

single-nucleotide polymorphisms (SNPs), and diabetes in larger populations. Cumulatively, these studies have provided insight into the genetic architecture of diabetes [237].

Prior to the GWAS era, the relatively high degree of familial clustering among patients with T1D already suggested a genetic contribution to the disease. For instance, the prevalence of T1D is increased by 6% in children with affected first-degree relatives [240]. The first and strongest genetic determinants, accounting for approximately 50% of genetic susceptibility, map to the human leukocyte antigen (HLA) region on chromosome 6p21.31 [241]. This region contains over 200 identified genes, some of which are involved in the immune response. Among the three groups, HLAs corresponding to class II, in particular the haplotypes HLA-DR3-DQ2 and HLA-DR4-DQ8, are present in about 90% of children with T1D. HLA class II molecules are cell surface receptors that present antigens to T-lymphocytes [242]. Interestingly, certain HLA haplotypes, like DR2-DQ6, have however shown to be protective against T1D, even in the presence of disease-associated autoantibodies [241]. Apart from the highly polymorphic HLA class II genes, other genes have been associated with an increased risk of T1D. The most relevant are: insulin (*INS*), protein tyrosine phosphatase-non-receptor type 22 (*PTPN22*) and cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*) [240,243–245]. Recent GWAS have not only confirmed the above-mentioned risk genes but also identified more than 60 other susceptibility loci. Novel regions associated with T1D predisposition harbour for example the *UBASH3A*, *BACH2*, *IL27*, *IL2RA* or *ERBB3* gene loci [246]. However, only very few loci have been studied in more detail to dissect the underlying disease mechanism. Several of the T1D risk genes can be linked to immune and inflammatory pathways. Furthermore, studies have pointed out that these genes are directly or indirectly interconnected. This applies for example to BACH2 and PTPN2, where BACH2 in crosstalk with PTPN2 modulates proinflammatory cytokine-induced apoptotic pathways in pancreatic β-cells [247].

In T2D, genetic risk loci appear more scattered across the genome rather than concentrated to one essential region, like the HLA region in T1D. Moreover, the predicted heritability varies from 25% to 80% across different studies and the prevalence of T2D differs widely among ethnical groups. Nevertheless, the lifetime risk of developing T2D is 40% for individuals with one affected parent and almost 70% if both parents have T2D [237]. Initial linkage analysis and candidate gene approaches have led to the discovery of relevant T2D-associated loci, namely PPARG and TCF7L2 [237,248]. TCF7L2 encodes a TF that is essential during Wnt signalling pathway and different studies implied that the intronic risk variant rs7903146 impairs β-cell function and insulin secretion, alters proglucagon gene expression and action of incretins–gut-released hormones stimulating insulin secretion in response to nutrient intake [249,250]. Moreover, several GWAS linked reproducible different

variants in TCF7L2 to T2D [239,251]. Another variant that is repeatedly associated with T2D in certain ethnic groups is the Pro12Ala polymorphism in PPARG encoding the nuclear receptor PPAR-γ [252]. This receptor, highly expressed in the adipose tissue, is involved in adipogenesis and the molecular target of thiazolidenediones, a widely used class of anti-diabetic drugs [253]. Today, 131 GWAS for T2D are listed in the NHGRI-EBI catalog (last data release on 2019-09-24). These identified several hundred susceptibility loci, which are related to distinct diabetic traits, like loci associated with decreased insulin secretion and fasting hyperglycaemia, loci which primarily affect insulin sensitivity, or those which influence insulin processing and secretion. On top of identifying novel putative risk loci, GWAS have implicated new pathways in the development of diabetes. One of the most illustrative examples was the finding of a variant (rs13266634) in the β-cell zinc transporter ZnT-8 (encoded by SLC30A8). Shortly after its publication, *in vitro* and SLC30A8 null mouse studies confirmed that coding variation in SLC30A8 can impair insulin secretion [254,255]. Despite the large number of variants discovered using GWAS, these only explain 10% of T2D heritability. This is likely due to the presence of common variants that have a small effect and/or rare variants that are not tagged by common SNPs. The majority of the loci identified through GWAS have not been studied yet [255]. There is a clear need to define the molecular bases by which risk variants contribute to diabetes susceptibility. Further, most variants map within non-coding regions, thus reinforcing the fundamental quest to characterize the biological relevance of non-coding regions and integrate other omics data.

1.2.2.2 The genetics of monogenic diabetes

In very rare cases, named monogenic diabetes, a single mutation in one gene is sufficient to cause diabetes. The two major forms of monogenic diabetes are MODY and NDM, which notably result from mutations in TFs or other proteins that regulate endocrine pancreas development or function [64,256]. Up to date over 30 genes have been linked to monogenic diabetes, yet many patients remain misdiagnosed [66]. Overlapping clinical features with common forms of polygenic diabetes, make diagnosis challenging. On top of this, the clinical phenotype can vary substantially among patients with the same mutation. Therefore, genetic testing should be done if monogenic diabetes is suspected. This does not only help in confirming the diagnosis but also in predicting disease course, defining risk for relatives and determining treatment [257].

The most common form of monogenic diabetes is MODY, first described in 1974 and nowadays representing a heterogenous group of diabetes, inherited in an autosomal-dominant fashion and typically diagnosed before the age of 25 years [64,258]. Patients generally have heterozygous mutations, and the penetrance can vary immensely, even among family members. Mutations causing MODY have been reported in at least 14 genes, which

are summarized in table 1 [66]. Among these mutation in genes encoding hepatocyte nuclear factor 4α (HNF4α -MODY1), glucokinase (GCK-MODY2) and hepatocyte nuclear factor 1α (HNF1α-MODY3) account collectively for approximately 85% to 90% of all MODY cases [259]. The TFs HNF4α and HNF1α regulate the expression of many genes essential for normal development and function of the liver, kidney, gut and pancreas [260–263]. Moreover, in hepatocytes HNF4α is a key activator of HNF1α, which in turn induces the expression of a large set of genes involved in glucose, cholesterol and fatty acid metabolism [262]. Conversely, in pancreatic β-cells HNF4α is a direct target gene of HNF1α. Recent studies in mice and human β-cell models emphasized the role of HNF1α in controlling β-cell function and growth by regulating glucose transporter 2 (GLUT2), pyruvate kinase, hepatocyte growth factor activator and collectrin [264–266]. Heterozygous mutations in HNF1α are more commonly found in MODY patients than HNF4α mutations. Over 400 distinct mutations in HNF1α have been reported in over 1,200 families [261,267]. These have been found all across the gene from the promoter to 3'UTR region, thus affecting the N-terminal dimerization -, the DNA-binding -, and the C-terminal transactivation domain of HNF1α. The highest mutation rate is in the HNF1α dimerization domain [261]. Haploinsufficiency or dominant negative forms of either HNF1α or HNF4α lead to progressive β-cell dysfunction [268–270]. This is manifested as mild hyperglycaemia in childhood and as diabetes during early adulthood. Patients with HNF1α and HNF4α mutations are sensitive to sulfonylureas, an oral antidiabetic drug, and can be treated long-term at low doses. In cases of sever decrease in β-cell insulin production, it may be necessary to switch to insulin therapy [66].

GCK-MODY is another common MODY form with mutations in 1 in 1000 individuals. *GCK* encodes the glucokinase enzyme, which phosphorylates glucose to glucose-6-phosphate during the first step of glycolysis [66]. Thus, *GCK* acts as a key glucose sensor and regulator of insulin release by pancreatic β-cells. Several mutations in *GCK* have been identified leading to both hyperglycaemia and hypoglycaemia [271]. Heterozygous loss-of-function mutations are associated with mild hyperglycaemia due to an elevated glucose threshold for insulin secretion [66]. Except during pregnancy, treatment of hyperglycaemia in GCK-MODY is generally not required since microvascular complications are extremely rare in these patients [272]. Mutations in *GCK* can also cause NDM [273].

NDM presents within the first 6 months of life and can persist throughout life (permanent NDM [PDNM]) or disappear during infancy (transient NDM [TNDM]). However, half of patients with TNDM experience relapse of diabetes during adolescence [274]. TNDM is often characterized by intrauterine growth retardation and caused by genetic abnormalities leading to overexpression of paternally expressed genes in the imprinted region of chromosome 6q24 [275]. This is due to uniparental paternal disomy, a paternally inherited duplication, or a maternal methylation defect [278]. For example, several patients with TNDM carry recessive

mutations in the *ZFP57* gene, which is critical for the maintenance of methylation imprints during development [279]. The underlying mechanism by which 6q24 region abnormalities lead to TNDM remains unclear, but the locus contains two expressed genes, *PLAGL1* and *HYMAI* [278]. PLAGL1 regulates cell cycle arrest, apoptosis and the expression of PACAP1, a potentiator of glucose-induced insulin secretion [278].

Table 1: List of MODY subtypes [64,66,260,276,277]**.**

Type	Gene name	% of all MODY	Pathophysiology	Other clinical features
1	*HNF4α*	5-10%	Progressive β-cell dysfunction	TNDM common; low triglycerides
2	*GCK*	30-50%	Glucose sensing defect	Mild, stable fasting hyper-glycaemia; typically asymptomatic; diagnosis often incidental
3	*HNF1α*	30-65%	Progressive β-cell dysfunction	TNDM in some cases; renal glycosuria
4	*PDX1*	1%	β-cell dysfunction	Pancreas agenesis and PNDM in homozygosity; Overweight/obesity in some cases
5	*HNF1B*	<5%	β-cell dysfunction	Pancreatic hypoplasia; renal/ genitourinary malformations; low birth weight
6	*NEUROD1*	<1%	β-cell dysfunction	Overweight/obesity in some cases; moderate to severe β-cell dys-function; NDM in some; neurological abnormalities
7	*KLF11*	<1%	β-cell dysfunction	Similar to T2D
8	*CEL*	<1%	Pancreas endocrine and exocrine dysfunction	Pancreatic atrophy (exocrine pancreatic insufficiency); fibrosis & lipomatosis
9	*PAX4*	<1%	β-cell dysfunction	Ketoacidosis-prone
10	*INS*	<1%	Mutation in insulin gene	Diabetes wide clinical spectrum; NDM in some cases
11	*BLK*	<1%	Insulin secretion defect	Overweight/obesity in some cases
12	*ABCC8*	<1%	ATP-sensitive potassium channel dysfunction	PNDM in homozygosity; TNDM in heterozygosity
13	*KCNJ11*	<1%	ATP-sensitive potassium channel dysfunction	Similar to MODY1 and MODY3; NDM in homozygosity
14	*APPL1*	<1%	Insulin secretion defect	Overweight/obesity in some cases

Mutations in *KCNJ11*, *ABCC8* and *INS* are associated with both TNDM and PNDM [280]. Activating heterozygous mutations in the genes encoding the β-cell ATP-sensitive potassium (K_{ATP}) channel are the most common cause of PNDM. KCJN11 encodes Kir6.1 and ABCC8 encodes SUR1, the two subunits of the K_{ATP} channel. This channel is an octameric complex, consisting of four pore-forming Kir6.2 subunits and four outer SUR1 regulatory subunits [66,281]. Further, this channel directly links glucose metabolism to insulin secretion by closing in response to ATP. Under physiological conditions, glucose uptake by the pancreatic β-cells through GLUT2 transporter, promotes glycolytic production of ATP. The increase in ATP/ADP ratio causes closure of the K_{ATP} channels, preventing potassium efflux. This in turn, leads to depolarization of the β-cell membrane, followed by influx of calcium through voltage-gated calcium channels and ultimately results in the exocytosis of insulin-containing granules from the β-cells [281]. Mutations in either gene reduce the ATP sensitivity of the K_{ATP} channel, thus impairing closure of the channel and thereby preventing glucose-induced insulin secretion [282]. The majority of these mutations are missense mutation that cause single amino acid substitutions [283]. Apart from the pancreas, K_{ATP} channels are also expressed in other tissues, like neurons, brain and muscle. As a consequence, some patients with KCNJ11 mutation show marked developmental delay, muscle weakness, and epilepsy [284]. Patients with KCNJ11- and ABCC8-dependent NDM can be successfully treated with sulfonylurea tablets rather than within insulin therapy. Sulfonylureas bind to the SUR1 subunits and close the KATP channel in an ATP-independent manner [66]. Apart from the above-mentioned genes, many more gene mutations have been reported in NDM. These include key pancreatic TFs, such as *PDX1*, *PTF1a*, *NEUROD1* and *HNF1β* [273].

Advances in molecular genetic analyses have led to the identification of several genes associated with clinically distinct forms of monogenic diabetes. Even though monogenic forms of diabetes are highly uncommon overall, they have greatly contributed to our current understanding of the pathogenesis of diabetes. Importantly, they represent invaluable models to study defects in pancreatic islet cell development and insulin secretion directly in a human *in vivo* context [65]. Furthermore, GWAS have expanded the repertoire of susceptibility loci associated with common T1D and T2D diabetes. However, functional studies to dissect the role of the identified variants and define their biological function have been challenged by the lack of appropriate human β-cell models. Therefore, even in the era of GWAS, monogenic diabetes continues to provide a platform to interpret GWAS data and potentially identify novel gene candidates crucial for β-cell development and function [65,285,286]. Moreover, latest GWAS revealed that several monogenic diabetes genes, including *KCNJ11*, ABCC8, GCK, HNF1α and HNF4α, are linked with common T2D. For example, Nielsen et al. and others demonstrated that the KCNJ11 E23K gene polymorphism is associated with an increased risk of T2D [287].

Taking together, monogenic diabetes offers excellent models to unravel targets of β-cell development and function in humans. New insight gained from these studies can help in defining genetic factors that increase T1D/T2D susceptibility and instruct translational efforts towards new treatment of diabetes.

1.3 Human models to study pancreas development in health and disease

Studies of human pancreas development are generally hindered by the limited accessibility to early human fetal tissues. Thus, our current understanding of mechanisms regulating pancreas development and function has been largely derived from animal models, including zebrafish, rats and mice [203,288]. However, species differences are present and recent studies have more and more highlighted the need for human models [289]. Advances in the stem cell and genome editing fields have paved the way to recapitulate normal pancreas development and to model disease pathways in a human context [290]. These novel technologies, including the generation of human induced pluripotent stem cells (iPSCs) and their directed differentiation toward pancreatic β-cells, as well as CRISPR-Cas9-mediated genome editing will be discussed in the following chapter [291]. Moreover, novel insights gained from these pancreatic human models will be outlined.

1.3.1 Human induced pluripotent stem cells

In 1962, Sir John Gurdon provided the first evidence that somatic cells can be reprogrammed into a pluripotent embryonic state by implanting a donor nucleus from a somatic cell into an enucleated oocyte which, after stimulation, developed into an organism. This method, named somatic cell nuclear transplantation (SCNT), challenged the previous dogma that embryonic development from immature to differentiated cells is unidirectional and irreversible [292]. Since the initial experiments in *Xenopus laevis*, SCNT has been successfully used to clone mice, pigs and the famous Dolly sheep, among many others [293–295]. Mouse ESCs and more recently human ESCs were generated, along with the development of the cell-cell fusion technique, which confirmed the feasibility to revert the potency state of somatic nuclei by either transfer to an oocyte or fusion with a pluripotent cell [296,297]. These findings greatly contributed to our understanding of the appropriate culture conditions and TFs required for the maintenance of pluripotency [298]. Moreover, ESCs with their infinite self-renewal capacity, as well as their ability to differentiate into every adult cell type, opened new avenues for developmental studies, disease modelling and drug screening [299,300]. However, ESCs are derived from the inner cell mass of pre-implantation blastocysts, therefore raising ethical issues related to embryo destruction and safety concerns due to immune rejection of non-autologous ECSs [300–302].

The discovery of induced pluripotent stem cells (iPSCs) by Shinya Yamanaka and Kazutoshi Takahashi in 2006 was a major breakthrough in the stem cell field. They identified four TFs, Oct4, Sox2, Klf4 and cMyc (together termed Yamanaka factors), which, when simultaneously overexpressed, are sufficient to convert mouse skin fibroblast into pluripotent stem cells [303,304]. Many other groups have since then confirmed that a wide range of somatic cells from mouse, human and other species can be successfully reprogrammed into iPSCs through retrovirus-mediated transduction of the Yamanaka factors [305–307]. The reprogrammed cells closely resemble ESCs in terms of morphology, proliferation capacity, differentiation potential, gene expression profile and epigenetic status [300,308]. Therefore, iPSCs provide an attractive alternative to ESCs and hold great promise for basic research and cell therapy. Over the years, additional reprogramming factors and delivery methods have been established to improve safety and efficiency of the procedure. Initial reprogramming methods utilized retroviral or lentiviral vectors for ectopic expression of the Yamanaka factors. These vectors ensure high reprogramming efficiency, harbour however the risk of causing chromosomal instability and tumorigenesis from insertional mutagenesis [305]. Thus, especially for cell therapy purposes, alternative delivery methods, like non-integrating viral vectors (adenovirus, Sendai virus), episomal plasmids (for example oriP/EBNA1), microRNAs and synthetic RNAs have been used to generate integration-free iPSC lines [305]. Furthermore, large-scale screenings have identified several chemical compounds, like E616452 (ALK5 inhibitor), valproic acid (histone deacetylase inhibitor), vitamin C, 5-azacytidine (DNA methyltransferase inhibitor) and CHIR99021 (GSK3-inhibitor), which enhance reprogramming efficiency or can even substitute individual/multiple Yamanaka factors [309]. In 2013, Hou and colleagues demonstrated for the first time complete chemical reprogramming of mouse embryonic fibroblast into iPSCs using a cocktail of seven small molecules, including VPA, CHIR99021, E616452, Tranylcypromine, Forskolin, DZNep and TTNPB [310]. Interestingly, chemically-induced iPSCs differ from TF-induced iPSCs in the way that they go through the formation of extra-embryonic endoderm-like cells [311]. So far, chemical induced human iPSCs have not been reported.

Since their discovery, numerous iPSC lines have been established and differentiated into derivatives of all three germ layers, like dopaminergic neurons (ectoderm), smooth muscle cells (mesoderm) and pancreatic β-cells (endoderm) [205,312,313]. As the iPSC technology offers the opportunity for generating patient-specific stem cells, they hold great promise for personalized regenerative cell therapy [314]. Indeed, in 2014, the first clinical trial using autologous iPSC-derived retinal pigment epithelium (RPE) was launched to treat a patient with age-related macular generation. Later on, the trial was reported to have improved patient's vision without immune rejection of the transplanted iPSC-RPE sheet [315]. Other clinical trials followed or are currently undertaken. However, the generation of GMP-grade autologous iPSC

lines is extremely laborious and cost intensive. To overcome this issue, Yamanaka initiated the establishment of a GMP-grade iPSC-bank in Japan (CiRA's iPSC bank for regenerative medicine) from homozygous, so called “HLA super-donors” [307]. Notably, in genetically homogenous countries, like Japan, it is estimated that about 10 allogeneic donor lines would be sufficient to cover about 70% of the Japanese population [316,317]. Nevertheless, the clinical translation of iPSC-derived cell products is still evolving and facing several challenges. Thus, it is not surprising that the majority of stem cell-based trials employ ESC-derivatives. Apart from their potential therapeutic applications, iPSCs represent an exciting human model to study diseases and conduct drug screenings. Pioneering studies have shown that iPSCs can faithfully recapitulate disease phenotypes *in vitro* when subsequently differentiated into disease-relevant cell types. Several groups already reprogrammed somatic cells from patients to study Lesch-Nhyan syndrome, Alzheimer disease and T1D. Moreover, iPSCs can provide an unlimited resource for any desired cell type, which otherwise would only be accessible post-mortem [318–320].

iPSCs have revolutionized regenerative medicine and developmental biology within a short period of time. They represent a unique opportunity to decipher pathophysiological pathways of various diseases and advance our understanding of developmental aspects at cellular level. Several challenges -low reprogramming and differentiation efficiency, contamination of the cell product with residual undifferentiated iPSCs, which can form unwanted teratomas- have to be overcome. In addition to technical hurdles, the basic biology of pluripotency needs to be further investigated [303,321]. But given the pace of progress, it seems likely that human iPSC will move from “bench to bedside” in near future.

1.3.2 Development and applications of CRISPR-Cas9 technology

Genome editing has remarkably improved over the last years and a variety of techniques evolved to introduce genetic changes in a site-specific manner, including Zinc Finger Nuclease (ZFN), Transcription-Activator Like Effector Nucleases (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-Associated 9 (CRISPR-Cas9) [322]. In recent years, the CRISPR-Cas9 system has received much attention and been exploited as a technology for manipulating the genome of virtually any organism. Initially, Ishino et al. described CRISPRs in *Escherichia coli* genome as unusual repetitive DNA sequences [323]. The significance of this structure was appreciated later when investigators realized that CRISPRs contain viral sequences and identified their associated Cas genes. Shortly thereafter, it was established that the CRISPR-Cas system plays a role during adaptive immunity in diverse species of bacteria and archaea. Numerous Cas effector proteins have been reported to protect the host from invading nucleic acids [324]. At present, the CRISPR-

Cas system is classified into two groups, based on their Cas effector proteins, which are then further subdivided into six types (I-VI), depending on their signature genes [325]. The class I system predominantly occurs in bacteria and archaea. However, class II systems have been mostly adapted and modified for genome engineering [325–327].

Jennifer Doudna and Emmanuelle Charpentier demonstrated in 2012 that the purified Cas9 from *Streptococcus pyogenes* (SpCas9) could be guided by CRISPR RNAs (crRNAs) to introduce *in vitro* a site-specific double-strand break (DSB) into target DNA. Furthermore, they highlighted the usage of CRISPR-Cas9 as a genome editing technology, by showing that a single guide RNA (sgRNA) could be engineered by fusing a crRNA containing the targeting guide sequence to a transactivating CRISPR RNA (tracrRNA) that facilitates DNA cleavage by Cas9 [326,328]. Afterwards Feng Zhang and colleagues successful edited human and other mammalian cells using CRISPR-Cas9 [329,330].

The basic principles of this technology are depicted in figure 3. Briefly, the CRISPR-Cas9 system consists of two parts: a Cas9 endonuclease protein and a synthetic sgRNA. The latter is composed of a “scaffold” sequence required for Cas9-binding and a 20 nucleotide long “spacer” sequence that defines the genomic target site. This sgRNA then complexes with Cas9. Site-specific cleavage occurs three base pairs upstream of the PAM sequence, which conforms to a 5’-NGG consensus sequence in the *S. pyogenes* Type II system. Thus, for accurate CRISPR-Cas9 genome editing a NGG-PAM sequence is necessary at target site. The generated blunt-ended DSB can then be repaired by either non- homologous end joining (NHEJ) or homology-directed repair (HDR) [324,326,327]. The error-prone NHEJ pathway can be exploited to generate gene knockouts based on the random insertion of indels (deletion/insertion). Instead, HDR can be employed to introduce/correct point mutations, create translocations or insert exogenous sequences (e.g. loxP sites, GFP-tags). To achieve this, a Cas9-mediated cut is generated adjacent to the position of interest, while providing a homologous donor template, like a short single-stranded DNA oligo (ssODN), carrying the intended nucleotide change [327,331]. Generally, NHEJ is favoured over HDR in mammalian cells and the latter mainly occurs in late S- or G_2-phase of the cell cycle [332]. Several approaches have been explored to improve HDR efficiency. These include reagents to arrest cell cycle at the S and G_2-phase, small molecules, which inhibit the NHEJ (e.g. SCR7, NU7441) or enhance the HDR pathway (e.g. RS-1, L755507), and modifications of the CRISPR-Cas9 components (e.g. fusion of Cas9 to dominant-negative 53BP1) [333,334].

A main limitation and clinical concern of the CRISPR-Cas9 system is the off-target effect, resulting from non-specific Cas9 cleavage at undesired genome sites [327,335]. To avoid these undesired off-targets, proper sgRNA design is crucial. In addition, mutant forms of Cas9 have emerged to further increase specificity. For instance, by mutating one of the two Cas9 nuclease domains, researchers created the Cas9 nickase (Cas9n) [336]. Nickases

generate single-strand breaks in DNA, but when paired with two sgRNAs, Cas9n effectively create DSBs with reduced off-target activity. In addition, an inactivated or dead Cas9 (dCas9) has been fused with cytidine deaminase to directly convert cytidine to uridine without the need for DSB and donor DNA template (referred to as “base editing”) [337]. Moreover, dCas9 has been fused to repressor/activator domains, epigenetic modifiers, fluorescent proteins and many more, to regulate gene expression, edit epigenome or visualize specific genomic sequences. Mandegar et al. established, for example, CRISPR interference (CRISPRi) to specifically and reversibly inhibit gene expression in human iPSCs and their derivatives. Here, a doxycycline-inducible dCas9 is fused to a KRAB repression domain [338]. Noteworthy, alternatives to Cas9, like Cas12a, additionally increase the versatile of the system by recognizing distinct PAM sequences and producing sticky ends after cleavage [324].

From its discovery as an adaptive immune system, the CRISPR-Cas system quickly emerged as a powerful, simple and inexpensive technology for genome editing. As outlined above, the CRISPR-Cas9 system has been adapted and modified extensively, thus holding great promise for applications far beyond gene editing [327]. Further advancement of the CRISPR-Cas9 technology, especially toward the therapeutic use, can be expected. Indeed, already in 2016, a patient with aggressive lung cancer was treated with CRISPR-Cas9 engineered T-cells (ClinicalTrials.gov: NCT02793856, Lu You) [339]. Moreover, studies addressing the safety, specificity and ethical implications of this tool will help in overcoming current challenges. Then, the CRISPR-Cas9 system has the potential to advance many more areas of biological and medical research.

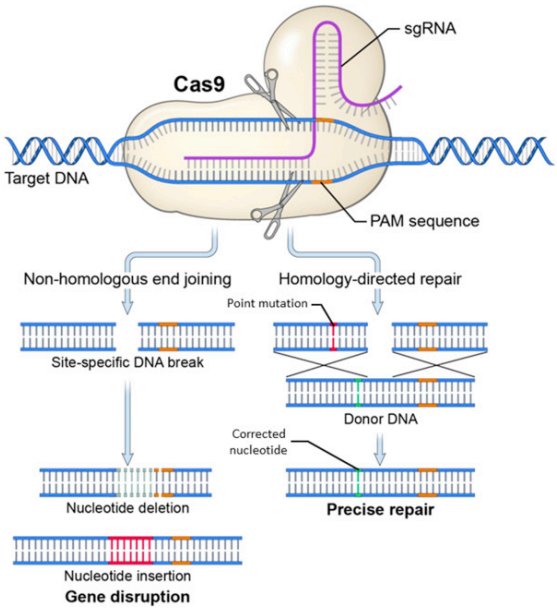

Fig. 3: The CRISPR-Cas9 nuclease system. After the sgRNA binds to the target site of genomic DNA, the Cas9 protein creates a DSB around the PAM site. DSBs in target genes can be repaired by NHEJ, leading to small sequence deletions and gene knockout alleles. Instead, precise sequence modifications (Knockin alleles) are introduced by HDR with the homology regions of DNA repair templates, either an ssODN or plasmid vector. Taken from Guitart et al., 2016.

1.3.3 Cell-replacement therapy for diabetes

The objective of cell-replacement therapies is to restore diseased or injured tissues by replacing lost cells with functional cells to re-establish normal function. This approach has gained widespread enthusiasm as a potential cure for common chronic diseases, including diabetes [17,17,340]. Here, loss (T1D) or dysfunction (T2D) of the insulin-producing β-cells results in impaired control of glycemia. Left untreated or managed poorly, diabetes leads to severe long-term complications that ultimately reduce life expectancy. To prevent this, management of diabetes aims at maintaining glycaemic control, which can be achieved through the exogenous administration of insulin. However, despite vast improvements in glucose monitoring (e.g. continuous monitor), insulin delivery (e.g. insulin pump) and innovation in insulin preparations (e.g. short & long-acting), patients still face the risk of acute hypo- and chronic hyperglycaemia [216]. Together with the increasing incidence of diabetes, there is a need to identify novel therapeutic approaches to restore normoglycaemia [13].

Current research efforts in regenerative medicine to cure diabetes include cadaveric islet transplantation, promoting endogenous β-cell regeneration, reprogramming of somatic cells toward a β-cell fate and administration of PSC-derived β-cells [341]. Among these, allogenic islet transplantation in T1D patients already provided a clear proof-of-concept for cell-therapy in diabetes. In combination with proper immunosuppressants, 25% to 50% of the most recent islet transplant recipients stayed insulin independent for up to 5 years [342,343]. However, this approach is extremely limited by the shortage of suitable donor tissue and detrimental side effects of immunosuppressive regimens [55]. To outline the necessity in donors, a single 68 kg diabetic patient requires transplantation of about 340 to 750 million islets; this is equivalent to two or three donors of pancreatic islets [344,345]. Therefore, other means of β-cell replacements are being explored to eliminate the reliance on donors and provide an abundant supply of β-cells. Furthermore, autologous β-cells would diminish the risk of immune rejection of the transplant.

One exciting approach is the direct differentiation of PSCs, preferable patient-derived iPSCs, into β-cells. The current state-of-the-art in β-cell generation from PSCs will be outlined in the next section. An interesting alternative to generate fully mature β-cells for clinical application is instead the direct transplantation of human PSC-derived pancreatic progenitors. Studies in T1D mice, proved that human ESC-derived pancreatic progenitors further differentiate and mature *in vivo* into β-cells and other endocrine cells. Moreover, the resulting cells produced human insulin and reversed diabetes in recipient mice. Also, survival and differentiation potential of the human ESC-derived pancreatic progenitor do not seem to be affected by encapsulation, supporting the potential use of such a system for the treatment of T1D [346]. Indeed, the first clinical trial has been launched by ViaCyte to test the safety and efficacy of encapsulated human ESC-derived pancreatic endoderm cells (NCT02239354).

Apart from the therapeutic potential this strategy holds, it also highlights that key *in vivo* factors regulating β-cell maturation are still elusive.

Another elegant alternative to human PSC-derived β-cell, is the direct lineage reprogramming of somatic cells into β-cells. In animal studies, pancreatic (e.g. α- and ductal cells) or even non-pancreatic cells (e.g. enteroendocrine cells, hepatocytes) could be directed to acquire a β-cell identity through the forced expression of relevant pancreatic TFs [55,347]. Significant efforts are undertaken to translate these findings into clinical applications. Both strategies–stem cell differentiation and somatic lineage reprogramming–hold great promise to realize regenerative medicine for diabetes.

1.3.4 Directed differentiation of human PSCs into pancreatic β-like cells

Stem cell-derived insulin-producing β-cells offer an inexhaustible supply of functional β-cells for cell-therapy, developmental studies and disease modelling. The current challenge in the field is to identify the appropriate signalling pathways and culture conditions to direct PSCs into *bona fide* human β-cells. Up to date, several protocols to differentiate human ESCs and iPSCs towards β-cells have been established [204,205,348–353]. All of them follow the common concept of recapitulating pancreatic development *in vitro* by directing cells through defined stages resembling DE, gut-tube endoderm, pancreatic endoderm and endocrine precursors to finally yield insulin-secreting β-cells [204,205,348]. This is conducted by sequential stimulation or inhibition of key signalling pathways with growth factors and small molecules. For instance, studies in vertebrate model organisms elucidated that two signalling pathways, the canonical WNT and TGF-β, are required for DE formation [41,192,197,354]. D'Amour et al. mimicked this *in vitro* by using WNT agonists (e.g. WNT3A or the GSK3β inhibitor CHIR99021) in combination with the TGF-β ligand Activin A to derive definitive endoderm cells from human ESCs in two-dimensional (2D) cultures [355,356]. These cells could be further differentiated into endocrine islet cells. However, the majority of the initial reported insulin-positive cells were polyhormonal, thus co-expressing other endocrine hormones (e.g. glucagon and/or somatostatin) and did not respond *in vitro* to glucose challenges–the hallmark function of mature β-cells. Nonetheless, these pancreatic progenitor cells were capable to mature upon transplantation into glucose-responsive β-cells *in vivo* [355].

To date, remarkable effort has been made to improve the differentiation efficiency of human PSCs to β-cells. Specifically, studies focused on optimizing the *in vitro* maturation step by systematically screening various soluble factors (small molecules, cytokines and growth factors) on pancreatic progenitors [204,205,348,351]. Novel *in vitro* differentiation protocols can convert human ESCs/iPSCs into glucose-responsive insulin-secreting β-cells, which

express mature β-cell markers and ameliorate hyperglycaemia in diabetic mice upon transplantation. Among others, the protocols from Russ et al., Rezania et al. and Pagliuca et al. show the most promising results (Fig. 4). These protocols have been reproduced and adapted by various research groups for numerous human PSCs lines, including iPSCs derived from diabetic patients [306,319,357]. Through six to seven stages, human PSCs are differentiated into cells that closely resemble primary human β-cells and are named henceforth β-like cells (equivalent to S7 cell in Rezania et al. and stem-cell derived β-cells [SC-β cells] in Pagliuca et al.). Unlike previous approaches, these optimized protocols include a three-dimensional (3D) culture step. In particular, Russ et al. and Paglicua et al. describe a suspension culture system with orbital shaker and spinner flasks, respectively. In contrast, Rezania et al. start differentiation in monolayer cells and changes to an air-liquid interphase culture at pancreatic endoderm stage. Overall, these protocols and others showed that 3D culture conditions improve differentiation into β-cells and promote maturation. Contrary to conventional 2D cultures, 3D platforms better recapitulate mechanical and intercellular cues present during normal islet development.

The protocol established by Russ et al. is the shortest one available based on simplified culture conditions of three weeks duration (Fig. 4). In more detail, Activin A together with WNT3a are used to induce the expression of typical anterior definitive endoderm markers, like *SOX17*, *CXCR4* and *FOXA2*. This is then followed by a short incubation with FGF7 in combination with inhibition of TGFβ to induce anterior-posterior patterning of the gut tube endoderm, marked by the expression of *HNF1β*. After reaching the gut tube stage, cell clusters are solely exposed to retinoic acid for two days, followed by EGF and FGF treatment until cells acquire a pancreatic progenitor fate biased towards the β-cell lineage. This results in the generation of about 80% of $PDX1^+/NKX6.1^+$ pancreatic progenitor (PP2) cells at day nine. In summary, the applied signals mimic the *in vivo* requirement for retinoic acid during pancreatic specification in the mice embryo and mesenchymal Fgf10 for maintenance and proliferation of pancreatic progenitors.

By contrast, the two other protocols established $PDX1^+/NKX6.1^+$ pancreatic progenitor cells (PP2) by using retinoic acid and FGF7 in combination with LDN and SANT-1 to inhibit BMP and SHH signalling pathways, respectively. The use of BMP and SHH inhibitors is based on previous observations in mice, showing that active BMP and SHH signalling prevent pancreas induction and promote the hepatic fate at the expense of the pancreatic one. Moreover, Rezania et al. supplemented the media with vitamin C at early stages (day 3-10) to prevent precocious expression of *NGN3*, a master regulator of pancreatic endocrine cell differentiation. However, Russ et al. illustrated that the addition of molecules to inhibit the BMP and SHH pathway at early stages leads to the premature expression of *NGN3*, which favours

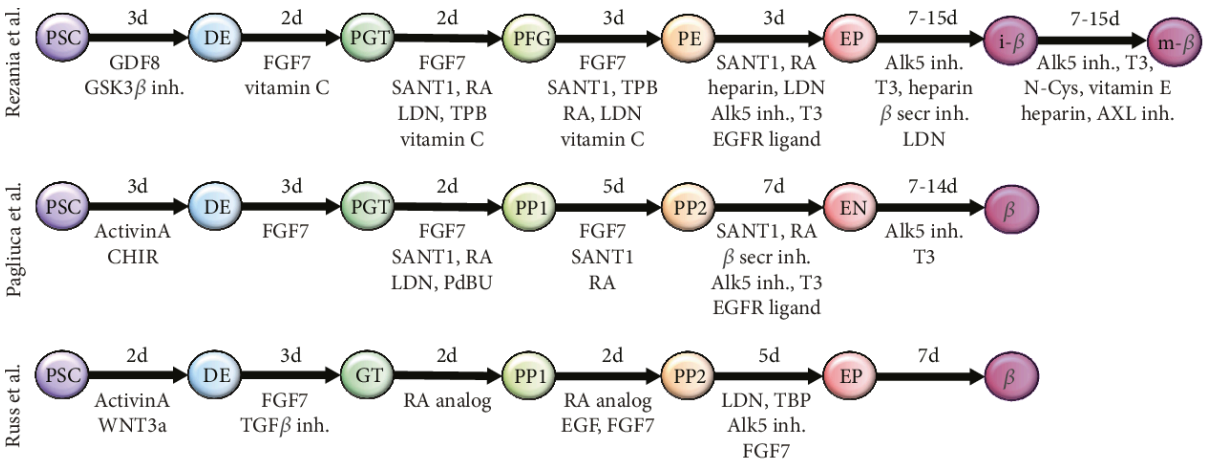

Fig. 4: Comparison of three *in vitro* pancreatic β-cell differentiation protocols. Schematic overview of stepwise pancreatic β-cell differentiation protocols described by Russ et al., Pagliuca and Rezania et al. [204,205,348]. Stage-specific modulation is achieved by the addition of distinct growth factors and small molecules. The duration of each stage is depicted in days. Specific markers are shown for essential stages. Pluripotent stem cells (PSCs), definitive endoderm (DE), primitive gut tube (PGT), posterior foregut (PFG), pancreatic endoderm (PE), pancreatic endocrine precursors (EP), immature β-cells (i-β), mature β-cells (m-β), PDX1$^+$ pancreatic progenitors (PP1), PDX1$^+$/NKX6.1$^+$ pancreatic progenitors (PP2), NKX6.1$^+$/C-peptide$^+$ cells (EN). Taken from Hohwieler et al., 2019 [358].

the generation of polyhormonal cells. Further, Russ et al. showed that the PP2 cells can be differentiated into endocrine cells by exposure to FGF7, TBP (protein kinase C activator), Alk5 inhibitor (TGF-β inhibitor), and LDN (BMP inhibitor). Inhibition of the BMP signalling pathway in the presence of other molecules efficiently recapitulated *in vitro* the transient expression of *NGN3* in PDX1$^+$/NKX6.1$^+$ progenitors. After endocrine commitment, downstream targets of *NGN3*, like *NKX2.2* and *NEUROD1*, were expressed in the differentiating cells. Finally, after a week in simplified culture conditions without growth factors, the EP cells gave rise predominantly to monohormonal insulin-producing cells that exhibited key feature of *bona fide* β-cells, including expression of key β-cell markers (*PDX1*, *INS*, *MAFA*, *MAFB*) and β-cell ultrastructure. Moreover, β-like cells remained glucose responsive after short-term transplantation and reduced blood glucose levels in murine model of diabetes. Also, only a small fraction of cells was polyhormonal (3%). Contrary, Rezania et al. exposed the pancreatic precursors (PE) to a combination of reagents, including a hedgehog inhibitor (SANT1), ALK5 inhibitor, BMP receptor inhibitor (LDN), and thyroid hormone (T3) to induce endocrine development, marked by the co-expression of PDX1$^+$/NKX6.1$^+$/NEUROD1$^+$. Continued exposure to an Alk5 inhibitor, T3, LDN, heparin and one Notch inhibitor, called gamma secretase inhibitor XX, resulted in a population of immature β-like cells, expressing *INSULIN* but without the mature marker *MAFA*. Although, the precise regulation of Notch signalling during pancreatic development in human is not fully understood, previous studies in mice have underscored the important role for Notch signalling [91,96,102]. While during early stages Notch signalling is required to maintain the pancreatic progenitor pool (Ngn3$^+$), a subsequent

reduction is essential for endocrine differentiation. Persistent elevated Notch activity induces *Sox9* expression and the Ngn3-inhibitor *Hes1*, thus promoting ductal differentiation [59,86,88,96]. In the context of *in vitro* differentiation, either inhibition of Notch signalling by gamma secretase inhibitors or inhibition of BMP and TGFβ signalling results in an increase insulin expression at later stages. During a final screen Rezania and colleagues identified an inhibitor of AXL, which in combination with ALK5 and T3, potently induced the expression of *MAFA* and thus the generation of mature β-like cells. After 43 days, the derived monohormonal β-like cells responded to glucose challenge with delayed kinetics and reversed diabetes following transplantation in immunodeficient mice. In line with their glucose responsiveness, the β-like cells expressed key genes encoding the insulin synthesis and secretion machinery (e.g. *INS*, *KCNJ11*, *PCSK2*, *PCSK1* and *ABCC8*) The differentiation protocol described by Pagluica et al. includes similar growth factors and small molecules as shown by Rezania et al., but finishes 10 days earlier. Here, the β-like cells responded to 2-3 successive glucose challenges *in vitro* by secreting insulin with a stimulation index similar to adult human islets.

Overall, the above-described multistage protocols accurately recapitulate key steps of human pancreas development and lead to a high percentage of endocrine progenitor cells ($NKX6.1^{+}/PDX1^{+}$). At the end of the differentiation at least 25% of the cells represent β-like cells marked by the expression of *PDX1* and *insulin* or *C-peptide*. Furthermore, the human PSC-derived β-cells resemble closely their *in vivo* counterpart–they express key markers for β-cell identity and function, and most notably sense and respond to changes in ambient glucose concentrations in static conditions. In addition, the simplified and scalable system described by Russ et al. provides an ideal platform to test novel intrinsic and extrinsic factors. Unlike other differentiation protocols, fewer growth factors and cytokines are applied thus reducing inconsistencies between different culture batches due to lot-to-lot variability. Also, the protocol is highly amenable for investigating the role and temporal coordination of other signalling pathways in the context of β-cell specification and maturation. Recently, the protocol was improved to mimic endocrine cell clustering by dissociation and reaggregation of immature β-like cells into enriched β-clusters [359]. Through the use of an $INS^{GFP/W}$ human ESC reporter line, the authors first optimized the previously published protocol and then sorted the $INS^{GFP/W}$ cells at day 20 by FACS. The GFP-sorted cells were reaggregated into clusters and cultured for an additional week, which permitted *in vitro* maturation of cells into β-like cells with enhanced functionality. Like adult islets, these newly generated β-like cells displayed dynamic insulin secretion, calcium response to glucose at comparable kinetics, and improved mitochondrial energization [359].

Despite the significant progress in differentiation protocols, further optimizations are required to minimize variations across different human ESC/hiPSC lines and create a homogeneous population of mature functional human β-cells. Then current protocols generate

a mixed population of cells that remain at a progenitor stage, do not follow endocrine commitment or even differentiate towards other undesired lineages. So far, protocols are lacking standardizations and only yield a small proportion of monohormonal β-like cells. These, unless a reporter human PSC line is employed, are difficult to purify because β-cell specific surface marker remain to be determined. Notably, the pancreatic secretory granule membrane major glycoprotein 2 (GP2) can be used as a surface marker to isolate pancreatic progenitors co-expressing *PDX1* and *NKX6.1* [360]. A recent study identified CD49a (also known as ITGA1) as a surface marker of β-cells, which allows magnetic sorting to a purity of 80%. In adult islets, CD49a is not expressed exclusively by β-cells but CD49 enrichment showed to deplete non-endocrine cells during differentiation, like enterochromaffin cells [20]. To also meet the demand in therapy, the process needs to be scaled up and should be conducted under xeno-free conditions, a criterion which is not met by most published protocols as they use animal sera to supplement media during differentiation [290]. Further, methods have to be developed to maintain β-cells in culture. Thus, studies to elucidate novel fate determinants will help in fine-tuning the activity of different signalling pathways to push pancreatic progenitors to differentiate into clinical grade β-cells. Once the appropriate developmental cues are discovered and the technical hurdles overcome, *in vitro* generation of β-cells in combination with new technologies to encapsulate and protect these cells will advance the treatment of diabetes.

1.3.5 Modelling human pancreas development and diabetes *in vitro*

Comprehensive studies in animal models, including zebrafish, rats and mice, provided a deep insight into pancreatic development and β-cell function. These investigations elucidated key TFs and genetic regulatory networks involved in pancreas formation and pancreatic progenitor differentiation into mature islet cells. Furthermore, several of these genes have been linked to monogenic or other types of diabetes through GWAS, thus confirming their role in human β-cell development. However, growing evidence supports the existence of significant differences between rodent and human pancreas. Therefore, human PSC models emerged as a resourceful tool to decipher developmental and disease mechanisms *in vitro*.

Over the last decade, several protocols have been established to differentiate human PSCs into distinct pancreatic lineages by recapitulating essential *in vivo* developmental events, which in combination with gene editing technologies have enabled to assess conservation between mice and humans [291]. Zhu and colleagues employed TALEN and CRISPR-Cas9-mediated gene editing to knockout eight pancreatic TFs in human ESCs. The study demonstrated that, like in mice, *HES1*, *ARX*, *RFX6*, *PDX1* and *NGN3* are required for

pancreatic development and cell differentiation in humans. In addition, the study identified a novel role of *RFX6* in regulating the formation of pancreatic progenitors and potentially divergent roles of some TFs in human and mice [361]. For instance, homozygous loss-of-function mutations in *PTF1A*, *MNX1* and *GLIS3* do not impair endocrine cell differentiation of human PSCs, while knockout mouse models show a reduced number of insulin-secreting β-cells. However, some phenotypes are not consistent across different studies. A key example is the requirement for *NGN3*. In mice, *Ngn3* is indispensable for the differentiation of all endocrine cell types and insulin^{+} cells are absent in *Ngn3*$^{-/-}$ mouse models [58]. However, patients carrying biallelic *NGN3* mutations suffer from impaired development of enteroendocrine cells and only a minority develop diabetes [362,363]. Also, these diabetic patients still possess residual β-cells as low levels of blood C-peptide can be detected. More recent studies using human PSC models addressed this divergence in NGN3 requirement with conflicting results. One study reported a complete lack of pancreatic endocrine cells upon *in vitro* differentiation of NGN3$^{-/-}$ human PSCs, whereas Zhu et al. detected residual insulin expression in NGN3$^{-/-}$ human PSC-derived β-like cells, whose functionality could not be assessed [361,364]. The underlying cause for this discrepancy is still unclear. Differences in differentiation protocols and genetic backgrounds of the human PSC lines could contribute to this phenomenon.

Human PSC-based models have helped in defining pathogenic pathways, as mouse models often failed to recapitulate disease phenotypes observed in humans. Noteworthy examples are mutations in the TFs GATA6, HNF1β (MODY5) and HNF1α (MODY3). For all of them, the patient phenotype is not mirrored in mice models. Three independent studies demonstrated that pancreatic endocrine progenitor formation is impaired in patient-like *GATA6* haploinsufficiency using human PSCs, while inactivation of one *Gata6* allele in mice does not cause apparent pancreatic defects [207,365,366]. *In vitro* differentiation of iPSCs from MODY5 patients (HNF1β$^{S148L/+}$) revealed impaired human β-cell development and function due to altered expression of various β-cell critical genes and reduced expression of the insulin gene activator *PAX6* [367]. Patients with MODY5 commonly exhibit pancreatic hypoplasia due to an autosomal dominant mutation in the *HNF1β* gene locus, while heterozygous *Hnf1β*$^{+/-}$ mice neither develop pancreatic hypoplasia nor diabetes [368]. Recently, Cardenas-Diaz et al. reported that ablation of *HNF1α* drives pancreatic endocrine differentiation towards a α-cell fate instead of β-cell through decreased expression of *PAX4*, a critical TF regulating β-cell development, which in turn leads to impaired insulin secretion and metabolism. In addition, they identified a human specific long non-coding RNA, LINKA, as a direct HNF1α target required for mitochondrial respiration [369]. Another study highlighted the use of human PSC models to elucidate organ-specific pathogenic mechanisms, otherwise underappreciated. For instance, diabetes in patients with STAT3 mutations was commonly attributed to an

autoimmune-mediated destruction of the endocrine cells. However, iPSCs derived from a NDM patient with an activating STAT3 mutation showed premature endocrine differentiation through *NGN3* upregulation based on the increased nuclear shuttling of the mutated protein. Correction of the STAT3 mutation with CRISPR-Cas9 rescued the premature differentiation phenotype [357]. Correcting patient-derived iPSCs is indeed a very powerful tool to assign causality to the mutation of interest. Moreover, genetically corrected cells represent a potential source for autologous β-cell replacement therapy. For example, iPSCs derived from patients with insulin gene mutation could restore effectively glucose-homeostasis in diabetic mice models upon gene correction.

The vast majority of diabetes in both adults and children is polygenic in origin. Thanks to GWAS, a large number of genes associated with T1D and T2D risk have been identified and modelled in human PSC models to some extent. The task is challenging because many of the disease-associated loci are present in non-coding DNA regions. In an elegant study, Zeng et al. established an isogenic human ESC-based platform to functionally evaluate the role of common T2D risk genes. B-like cells derived from human ESCs with biallelic loss-of-function mutations in the T2D risk genes *CDKAL1*, *KCNQ1* and *KCNJ11* exhibited impaired insulin secretion *in vitro* and *CDKAL1*$^{-/-}$ knockout cells were sensitive to glucolipotxicity. Further, a high-content chemical screen identified a candidate drug, which rescued mutant *CDKAL1* associated β-cell defects. Further studies showed that *CDKAL1*$^{-/-}$ knockout results in downregulation of the metallothionein gene, increasing sensitivity to endoplasmic reticulum stress [370].

T1D is associated with β-cell targeted autoimmunity but there is also evidence that intrinsic β-cell factors might contribute to disease pathogenesis. Several groups generated iPSC lines from individuals with T1D and differentiated them into β-like cells. However, phenotypic differences were only reported in β-like cells derived from fulminant T1D iPSCs, which showed increased apoptosis after cytokine treatment and altered expression of immune-related genes. Fulminant T1D is classified as idiopathic subtype of T1D and marked by the rapid loss of β-cells with less autoimmune contribution [319].

Finally, stem cell-based approaches can also be used to study diseases of the exocrine compartment of the pancreas. Although research efforts have mostly focused on endocrine cells, protocols are also available to differentiate human PSCs towards the exocrine-like lineages, such as acinar and ductal cells. For example, using this approach several groups modelled pancreatic insufficiency in genetic exocrine diseases, like Shwachman-Diamond Syndrome or cystic fibrosis [371,372]. Directed differentiation of human PSCs toward the pancreatic lineage is now a viable and attractive method to study human pancreas development and disease mechanisms. Also, novel genes and signalling pathways

involved in the pancreatic differentiation process, as well as their developmental time window, can be explored in human PSC models (Fig. 5). To effectively apply human PSC models in clinic, it is important to further refine and scale-up existing platforms. Together with 3D models, like organoids, and bioengineering approaches complex interactions between islet, endothelial and immune cells will help to more faithfully model pancreas development and diabetes. In summary, these technological advances are now beginning to enable large-scale diabetes modelling and drug screening to find new therapeutic interventions.

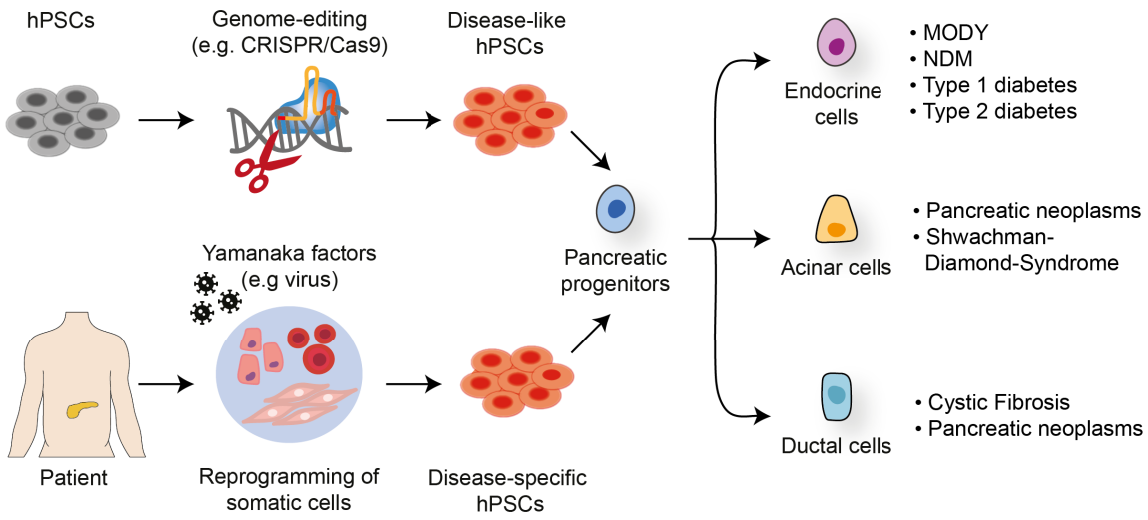

Fig. 5: Approaches to model genetic causes of pancreatic diseases. Disease-associated mutations/variants are introduced into human PSCs via genome editing or patient-specific iPSCs are generated via reprogramming of somatic cells. These approaches can be employed to study different endocrine and exocrine diseases. Human pluripotent stem cells (hPSCs), induced pluripotent stem cells (iPSCs), Maturity onset diabetes of the young (MODY), type 1 diabetes (T1D) type 2 diabetes (T2D).

2 AIMS OF THE STUDY

Diabetes is a group of metabolic diseases characterized by hyperglycemia and still a leading cause of mortality and morbidity worldwide. Currently, there is no cure for diabetes and therapeutic options are limited. Moreover, fundamental processes during pancreas development remain to be determined, hampering our understanding of the disease mechanisms. Thus, identifying novel regulators of β-cell development and function will not only advance our understanding of these biological processes but importantly help in devising novel strategies for diabetes therapy.

The overall aim of my PhD was to investigate novel putative disease-associated genes for diabetes and their role(s) in β-cell development. A set of new putative disease-causing variants was identified by next-generation sequencing of a unique cohort of patients with puberty-onset diabetes. I focused on variants found in the Histone deacetylase 4 (*HDAC4*), Glioma-associated oncogene homolog 1 (*GLI1*) and Glioma-associated oncogene homolog 2 (*GLI2*) genes. So far, mutations in these genes have not been associated with impaired β-cell differentiation or function. These gene candidates were prioritized for functional analysis based on patient phenotype and expression level in mouse pancreas progenitor cells. Moreover, I performed *in silico* analysis and functional studies in different model systems to identify those variants that are more likely of interest for pancreas development and β-cell differentiation. Finally, I selected one variant per gene candidate to be studied in human iPSC-based models of β-cell development. To this aim, I introduced the respective patient variants into a healthy iPSC line using CRISPR-Cas9-mediated genome editing. Employing CRISPR-Cas9-mediated genome editing in combination with human iPSC-directed β-cell differentiation, I assessed phenotypic differences between wild-type and patient-like cells for the expression of stage-specific markers at RNA and protein level. Ultimately, I expanded the phenotypic characterization of one patient variant in the GLI2 gene to shed light into the underlying disease mechanism.

3 MATERIAL AND METHOD

3.1 Molecular biology methods

3.1.1 Cloning of expression plasmids

To create expression plasmids and templates for RNA synthesis the open reading frame (ORF) of human *HDAC4* and its mutant variants *HDAC4* c.A680G and *HDAC4* c.G700A were amplified from pcDNA-HDAC4-FLAG using Ex-Taq DNA polymerase (Takara) with primers carrying restriction enzyme sites for EcoRI and XbaI. From here, the HDAC4 ORFs were directly cloned into the linearized (EcoRI and XbaI) pCS2^{++} expression vector (Table 2, 3). The HDAC4 expressing plasmids were a generous gift from Dr. Raile.

Table 2: PCR program.

Temperature	Time	Cycles
95°C	10 min	1
95°C	15 s	
55°C	60 s	25
72°C	15 s	
72°C	10 min	1
4°C	∞	

Table 3: RNA-synthesis.

Plasmid	Gene	Species	Restriction enzyme	Polymerase
mRNA synthesis				
pCS2^{++}-HDAC4 WT	*HDAC4*	Human	AscI	SP6
pCS2^{++}-HDAC4 c.A680G	*HDAC4*	Human	AscI	SP6
pCS2^{++}-HDAC4 c.G700A	*HDAC4*	Human	AscI	SP6
Synthesis of ISH probes				
pGem-xinsulin	*insulin*	*Xenopus laevis*	SalI	T7
pGem-xptf1a	*ptf1a*	*Xenopus laevis*	NotI	T7

3.1.2 *In vitro* transcription of mRNA

Plasmids expressing human *HDAC4 WT*, *HDAC4* c.A680G and *HDAC4* c.G700A ORF were linearized with AscI (NEB). Linearized DNA was extracted with equal amounts of phenol-chloroform-isoamyl alcohol and precipitated overnight with 2 Vol 100% EtOH, 10 µl NaAc (3 M) and 1 µl glycogen (20 mg/ml) at -20°C. The pellets were washed with 70% EtOH-DEPC, air-dried and resolved in 20 µl DEPC-H_2O. Linearized DNA was used as template for *in vitro* transcription of mRNAs using SP6-Message Machine Kit (Ambion) according to the manufacturer instructions (Table 4). The obtained mRNA was purified by phenol/chloroform extraction, precipitated with ethanol and dissolved in 20 µl H_2O. mRNA quality was quantified using NanoDrop™.

Table 4: Reverse transcription.

	volume
RNA	11 µl
DEPC-H_2O	2 µl
5x MMLV RTase buffer (Invitrogen)	4 µl
20 mM DTT (Invitrogen)	1 µl
10 mM dNTPs mix (Promega)	1 µl
RNAsin (Promega)	1 µl
MMLV RT (Invitrogen)	0.25 µl
Total volume	20.25 µl

3.1.3 *In vitro* transcription of digoxygen-labeled RNA

Plasmids coding for *insulin* and *ptf1a in situ* hybridization probes were linearized with SalI and NotI, respectively. Linearized plasmids were extracted with equal amounts of phenol-chloroform-isoamyl alcohol and precipitated overnight with 2 Vol 100% EtOH, 10 µl NaAc (3 M) and 1 µl glycogen (20 mg/ml) at -20°C. Pellets were washed with 70% EtOH-DEPC, air-dried and dissolved in 20 µl DEPC-H_2O. *In situ* RNA probes were *in vitro* transcribed using T7-polymerase (Roche) in the presence of transcription buffer (Invitrogen), DTT, digoxygenin-labeled NTPs (Roche) and RNasin (Promega) (Table 3). Quality of RNAs was validated by polyacrylamid gel electrophorese and quantified using NanoDrop™.

3.1.4 RNA isolation and cDNA synthesis

Injected and un-injected *Xenopus* explants were lysed at tadpole stage in RNA lysis buffer supplemented with 250 µl/ml proteinase K (Roche). Whole embryo lysates were carried out as internal control. Lysates were incubated for 30 min at 65°C, extracted with equal amount of phenol/chloroform/isoamyl alcohol and precipitated overnight with 500 µl 100% EtOH, 20 µl ammoniumacetat (10 M) and 1 µl glycogen (20 mg/ml) at -20°C. Pellets were washed with

70% EtOH-DEPC, air-dried and resuspended in 15 µl (explants) or 30 µl (whole embryo) DEPC-H_2O, respectively. All extracts were DNase treated at 37°C for 1 to 2 hrs. After DNAse treatment, 75µl DEPC-H_2O was added, RNAs were extracted as previously described. Ultimately, RNA pellets were dissolved in 11 µl DEPC-H_2O containing 0.1 µg random hexamer primers (Invitrogen) and 1 µg oligodT primers (Invitrogen), incubated for 4 min at 65°C and reverse transcribed according to the reaction mix summarized in table 5.

RNA lysis buffer: 20 mM Tris-HCl (pH 7.8), 1% SDS, 100 mM NaCl, 10 mM EDTA

In contrast, RNA of iPSCs and differentiated cell types was isolated using the High Pure RNA Isolation Kit (Roche). Samples were also treated with DNase to remove genomic DNA. Briefly, 3 µg of RNA extracted from cell culture samples were treated with 1-2 µL DNase Turbo (Ambion) for 30 minutes to 1 hr at 37°C. The RNA was purified through a phenol-chloroform extraction by adding 1 volume of phenol-chloroform-isoamyl alcohol to the sample and mixing both phases by vigorous shaking. Following centrifugation, the aqueous phase was transferred to a new tube. The RNA was precipitated at -20°C overnight with 1/10 volumes 3 M sodium acetate, 2 volumes ice-cold 100% ethanol and 20 µg glycogen. The following day, the samples were centrifuged for 15 minutes at 4°C and the pellets washed with 70% ice-cold ethanol. The RNA was resuspended in 20 µL DEPC-H_2O. 10 µL were used for RT and 10 µL for a control without reverse transcriptase (-RT control). The RT was carried out with the Transcriptor First Strand cDNA Synthesis kit (Roche). First strand reverse transcription was performed with 1.5 µg RNA using the Transcriptor First Strand cDNA Synthesis Kit. A mixture of random hexamers and oligo-dT primers was used for priming.

3.1.5 Quantitative Real-time Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was carried out in triplicates using the SYBR Green PCR Master Mix on ABI StepOne Plus system (Applied Biosystem) or the FastStart Universal SYBR Green Master Mix on LightCycle® (Roche). Results were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) *or acidic ribosomal phosphoprotein P0* (*36B4*) transcripts. For *Xenopus* samples the results were normalized to *ornithin decarboxylase* (*odc*). All reactions were performed with annealing at 60°C for 40 cycles. For undetectable transcripts, the cycle number was set to 40 for comparisons. Data were analysed using the REST randomization test or ΔΔCt method [373].

3.1.6 Genotyping of single cell-derived colonies

Genomic DNA was extracted from single cell-derived colonies in a 12-well plate. Once the culture reached confluence, genomic DNA was extracted from half a 12-well plate. The rest of the culture was cryopreserved. For genomic DNA extraction, cells were lysed for 3 hrs. at 60°C in 150 µl lysis buffer. The DNA was directly precipitated at RT for 30 min by adding 450 µl of precipitation buffer. Precipitated DNA pellets were washed 3x with 500 µl of 70% EtOH and resuspended in 45 µl resuspension buffer.

Genotyping PCR was carried out on genomic DNA extracted from iPSC clones. The PCR reaction contained 1 µL genomic DNA, 250 nM PCR primer mix, and 1x DreamTaqTM mix (Thermo Fisher Scientific), which contains TaqPolymerase, PCR buffer, 4 mM $MgCl_2$, 40 mM dNTPs and loading dye. The cycler programs are described in table 5. The primer combinations are described in table 6. All PCR reactions were carried out in a PCR cycler PTC-200 (MJ Research) or an Arktik thermal cycler (Thermo Fisher Scientific). PCR products of triplicates were combined, purified on agarose gels and Sanger sequenced.

Lysis buffer	10 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% Sarcosyl, 1 mg/ml proteinase K
Precipitation buffer	150 mM NaCl, 100% EtOH
Resuspension buffer	10 mM Tris-HCl pH 8.0, 10 mM EDTA

Table 5: Genotyping PCR program.

Temperature	Time	Cycles
95°C	5 min	1
95°C Tm (see Table 6) 72°C	30 s 1 min 1 min/kb	30
72°C	10 min	1
4°C	∞	

Table 6: Genotyping primer pairs.

Genotype	Primer name	Sequence (5' to 3')	Tm (°C)	Product size
HDAC4 c.A680G, HDAC4 c.G700A	HDAC4-mt_PCR-F	CATCGTGTGTGGCTTCTCGG	57	509
	HDAC4-mt_PCR-R	GCCCCTCAGTGTGGCAAAATC		
GLI1 c.C119G	GLI1-119_PCR-F	CCCAATCCTTCCTGAGACTTCC	55	492
	GLI1-119_PCR-R	CAAGCCAGGTCTTCCTGCCC		
GLI2 c.C4661T	GLI2-4661_PCR-F	GGAGGCCCCCCAGATTGAC	58	519
	GLI2-4661_PCR-R	GCACCAAAGGCTGGAAAGCAC		

3.1.7 Isolation of DNA from agarose gels

PCR products or digested DNA were resolved on 1.5% or 2.5% agarose gels. The corresponding bands were cut out and purified with the QIAquick Gel Extraction kit (Qiagen).

3.1.8 Primer design

Oligonucleotides were designed using NCBI's Primer BLAST. Primers were purchased from MWG Eurofin (Luxembourg). RT-qPCR primer sequences can be found in tables 9 to 12.

3.1.9 Site-directed mutagenesis

Site-directed mutagenesis was used to introduce the patient variant into the pBluescriptKS-GLI1 KT or the pCS2-MT GLI2 FL plasmid (Adgene), respectively. Moreover, mutagenesis was performed with the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent) following manufacturer's instructions.

3.2 *Xenopus* experiments

3.2.1 Obtaining and culturing Xenopus embryos

Animals were kept in standard conditions and manipulated according to the regulation of the local animal protection authority (Landesamt für Gesundheit und Soziales, Berlin).

Xenopus embryos were obtained and cultured according to Sive et al. [374]. In detail, ovulation was induced in adult *Xenopus* females by injection of 800 units of human chorionic gonadotropin (Sigma) into the dorsal lymph sac to promote spawning the night before egg collection. The next morning oocytes were collected into a clean petri dish by gentle massage of the female belly. To harvest testes for fertilization, a male frog was sacrificed by injection of 500 µl ethyl-3-aminobenzoate (Sigma) into leg musculature. Dissected testes were kept in 1x MBS solution (incl. 50 µg/ml gentamycin). For fertilization, a small piece of testis was homogenized in 1x MBS and submitted to the eggs. Eggs were kept at RT in 0.1x MMR until rotation of embryos was finished. At this stage, embryos were dejellied in cysteine solution for 2-4 min, washed in 0.1x MMR and stored at 14°C until further manipulated.

10x MMR (pH7.4):	0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5mM HEPES (pH 7.8), 0.1 mM EDTA
MBS:	0.1 M $CaCl_2$, 10 x MBS salts, 5 M NaCl
Cysteine solution, pH 8.0:	1 x MMR, 2% (w/v) cysteine

3.2.2 Manipulation of *Xenopus* embryos

Microinjection of *Xenopus* embryos was done according to Sive et al. [374]. Briefly, fertilized and dejellied embryos were transferred into 3.5% Ficoll (Sigma). Then 1 ng of mRNA were injected into two blastomeres of 4-cell stage *Xenopus* embryos using a microinjector. Injected embryos were kept overnight at 14°C in 3.5% Ficoll (Sigma), then transferred into 0.1x MMR and cultured until the desired stage at 16°C. mRNAs used for injection were human *HDAC4 WT*, human *HDAC4 p.H227R* and human *HDAC4 p.D234N*. Ultimately, embryos were fixed in MEMFA for 2 hrs. and stored in 100% MeOH or directly lysed for RNA extraction.

Ficoll:	3.5% Ficoll in 0.1x MMR
MEMFA:	0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde

3.2.3 Microdissection of *Xenopus* embryos

At early gastrula stage the vitelline membrane of *Xenopus* embryo was removed with tweezers and explants were dissected in 0.1% MMR using eyelashes. Microdissection was carried out to isolate dorsal and ventral endoderm for RT-qPCR analysis. Explants were cultured until tadpole stage in 0.75% MMR.

3.2.4 Whole-mount *in situ* hybridization of *Xenopus* embryos

Whole-mount *in situ* hybridization of *Xenopus* embryos was performed in 4 ml glass vials (BTC) on a nutator. After rehydration, *Xenopus* embryos were washed in PTW (0.1% Tween-20 in 1x DEPC-PBS) and treated with 10 µg/ml proteinase K for 3 min. Embryos were washed 2x in 0.1 M triethanolamin, treated 2x with 12 µl acidic acid and washed again in 1x PTW. Upon fixation in 4% PFA, embryos were washed 2x in 1x PTW and pre-hybridized for at least 1 hr at 60°C. Hybridization was done overnight with 1 ng/ml *in situ* probe at 60°C. Afterwards, embryos were washed in 2x SSC at 60°C, treated with 20 µg/ml RNase A and 10 units/ml RNase T1 at 37°C and then washed in 0.2x SSC. Embryos were washed in 1x MAB and 1x MAB/BMB. Blocking was done in 1x MAB/BMB/GS. Incubation of anti-digoxigenin-antibody (Roche) was done overnight at 4°C. Embryos were washed 5x in 1x MAB for 1 hr

each. Finally, after 2 washes in AP-buffer, colour reaction was carried out using BMPurple solution (Roth) in the dark. Colour reactions was stopped by washing embryos in 1x PBS. Finally, embryos were re-fixed in 4% PFA. For sectioning, embryos were embedded in gelatin and sectioned using a vibratome (Leica VT1000S).

Hybridization buffer:	50% formamide, 5x SSC, 1 mg/ml torula RNA, 100 µg/ml heparin, 1x denhardt‘s solution, 0.1% Tween-20, 0.1% CHAPS, 10 mM EDTA
1x MAB/BMB/GS:	1x MAB, 2% blocking reagent (BMB, Roche), 20% goat serum (GS, Gibco)
MAB, pH 7.5:	100 mM maleic acid, 150 mM NaCl
100x denhardt‘s solution:	2% BSA, 2% polyvinylpyrrolidone, 2% ficoll-400
20x SSC, pH 7.0:	3 M NaCl, 300 mM sodium citrate
AP buffer:	100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween- 20

3.3 Cell culture methods

3.3.1 Culture conditions

3.3.1.1 Culture of mouse Min6 insulinoma cells

Mouse Min6 insulinoma cells were grown in Dulbecco's modified Eagle medium (DMEM) (Life Technologies) supplemented with 15% fetal bovine serum (FBS), 1x non-essential amino acids (Life Technologies), 1% penicillin/streptomycin (Pen/Strep) and 0.5 µM beta-mercaptoethanol (Life Technologies). Medium was changed every second day and cells were passaged at 1:3 or 1:5 ratio.

3.3.1.2 Cell culture of human embryonic kidney (HEK) 293T cells

HEK293T cells were cultured in DMEM medium supplemented with 10% FBS and 1% Pen/Strep at 37°C, 5% CO_2.

3.3.1.3 Culture of human iPSCs

The human iPSC lines BiH005-A and CRISPRi were kindly provided by Dr. Diecke (Pluripotent Stem Cell Core, MDC) and the human iPSC line iXM001 by Dr. Kühn

(Transgenics, MDC). The human iPSC lines iXM001 and BiH005 were maintained on Geltrex-coated (Invitrogen) plates in E8 media (STEMCELL Technologies). The medium was changed daily, and cells were passaged every 3 days as cell clumps or single cells using 0.5 mM EDTA (Invitrogen) or Accutase (Invitrogen), respectively. Instead, the human iPSC line CRISPRi was maintained on Geltrex-coated (Invitrogen) plates in mTESR medium (STEMCELL Technologies) and passaged every 3 days as single cells using Accutase (Invitrogen). Medium was supplemented with 10 µM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Sigma) when iPSCs were thawed or passaged as single cells. iPSCs were cryopreserved in BambankerTM freezing medium (NIPPON Genetics) and stored in liquid nitrogen until further used. All cell lines tested negative for mycoplasma contamination which was carried out routinely.

3.3.2 Transfection of Min6 and HEK293T cells

For over-expression experiments in Min6 cells, 2.0 x 10^6 cells were transfected with 2 µg DNA using the transfection reagent nucleofector kit and the Nucleofector II Device (program G16) according to manufacturer's instructions. HEK293T cells were transfected using Polyethylenimine "Max" (PEI) (Polysciences). During transfection HEK293T cells were kept in OptiMEM medium (Invitrogen).

3.3.3 Luciferase assay

For luciferase assays, the dual luciferase reporter assay kit (Promega) was used. HEK293T cells were seeded at 1.3 x 10^6 cells in 12-well plates and transiently co-transfected in duplicates with 0.5 µg of constitutively active wild-type or mutant GLI1/GLI2 expressing plasmids together with 0.5 µg of GLI-responsive Firefly luciferase reporter construct (8x3'Gli-BSδ51LucII) and 0.05 µg of a constitutive Renilla luciferase reporter (pRL-SV40) construct. Protein lysates were prepared 48 hrs. after transfection. *Luciferase* and *Renilla* activity was quantified using the dual reporter assay kit (Promega) according to the manufacturer's instructions using an Infinite 200 Pro-luminometer (TECAN). Luciferase assay experiments were repeated three times on independent samples. As negative controls, the mutated version of the GLI-luciferase reporter construct (8xm3'Gli-BSδ51LucII) was used, as well as a GFP-expressing construct. Firefly/Renilla activity ratio was then calculated for each sample. The luciferase reporter constructs were kindly provided by Dr. Hammes-Lewin (MDC).

3.3.4 Differentiation of pluripotent iPSCs into β-like cells

Differentiation was carried out following a 21-day protocol described previously by Russ et al. [205]. Briefly, iPSCs were dissociated using Accuatase (Invitrogen) and seeded at a density of 5.5 x10^6 cells per well in ultra-low attachment 6-well plates (Corning) in 5.5 ml E8-home medium supplemented with 10 µM ROCK inhibitor (Sigma), 10 ng/ml Activin A (R&D) and 10 ng/ml Heregulin-b1 (PeproTech). Plates were placed on an orbital shaker at 100 rpm to induce sphere formation at 37°C in a humidified atmosphere containing 5% CO_2. Definitive endoderm differentiation was induced after 36 hrs. and cell clusters were subsequently differentiated into β-like cells by exposure to the appropriate medium. In addition, differentiation was carried out following a 27-day protocol described previously by Rezania et al. [204]. The differentiation was also carried out in suspension on an orbital shaker.

3.3.5 Generation of clonal iXM001 patient-like mutant lines

Patient-like iPSC lines were generated using the DOX inducible iCRISPR system previously established by Yumlu et al. [331]. Briefly, sgRNAs targeting the site of mutation were designed using the CRISPOR website (http://crispor.tefor.net/) and cloned into the pU6-(BbsI)sgRNA_CAG-venus-bpA vector by *BbsI* restriction enzyme overhangs. The sgRNA expression vector was a kind gift of Dr. Kühn (Transgenics, MDC). The respective point mutations were introduced through HDR using a 119 bp long ssODN. One day prior to transfection cells were seeded at 0.2 x 10^6 cells per well of pre-coated 6-well plate as single cells. For targeting, cells were transfected with 0.5 µg sgRNA vector and 30 pmol ssODN

Table 7: CRISPR-Cas9 gene editing in human iPSCs.

Target	sgRNA (5' to 3')	ssODN (5' to 3')
HDAC4 c.A680G	CGTCGTACATTCCCAGGACC GTCGACCTCCTATAACCACC	GCACAGTTCCCTTGACCAGAGTTCTCCACCCCAGAG CGGAGTGTCGACCTCCTATAACCGCCCGGTCCTGG GAATGTACGACGCCAAAGATGACTTCCCTCTTAGGA AAACAGGTCAGT
HDAC4 c.G700A	CTTTGGCGTCGTACATTCCC CCTAAGAGGGAAGTCATCTT	GTTCTCCACCCCAGAGCGGAGTGTCGACCTCCTATA ACCACCCGGTTCTGGGAATGTACAACGCCAAAGATG ACTTCCCTCTTAGGAAAACAGGTCAGTGCCCAGGCA TGCTGTGGGCC
GLI1 c.C119G	ATCCCACATCCTCAGTCCCG	CTTTGGGGTCCAGCCTTGTGGTCCCCATGACTCTGC CCGGGGTGGGATGATTCCACATCGTCAGTCCCGGG GACCCTTCCCAACTTGCCAGGTGAGAGTCCCCATAT TGCTACCTGATT
GLI2 c.C4661T	TCCATCCCCGCAGGCATCAG	CCACAGCCTCTCCCAGAACTCCTCCCGCCTCACCAC CCCCCGAAACTCCTTGACCCTGCTCTCTATCCCCGC AGGCATCAGCAACATGGCTGTCGGGGACATGAGCT CCATGCTCACCA

using Lipofectamin 3000 (Invitrogen) according to manufacturer's instructions. The next two days cells were feed with medium supplemented with 1 µg/ml DOX and then live-sorted for Venus. After FACS, single cells were expanded for 3 weeks to form colonies that were screened for recombination with the donor template. Positive clones were validated by Sanger sequencing. Sequences of sgRNAs, ssODNs and genotyping primers are listed in table 7.

3.3.6 Generation of CRISPRi *HDAC4* iPSC lines

Two sgRNAs were designed using the CRISPOR website to target the transcription start site of the *HDAC4* gene. Designed sgRNAs (Table 8) were phosphorylated, annealed and cloned into the sgRNA-expression (pgRNA-CKB) vector employing a BsmBI ligation strategy. The CRISPRi cells were nucleotransfected with the generated sgRNA-expression vector using Amaxa's protocol. Following transfection, the iPSCs were seeded in a single 6-well plate in mTeSR supplemented with ROCK inhibitor (Sigma). Blasticidin selection (10 µg/ml) was applied 24 hrs. post nucleofection until stable clones appeared. These were pooled and further passaged in the presence of Blasticidin and ROCK inhibitor (Sigma). Knockdown efficiency was analysed by RT-qPCR after treating stable bulk cultures with DOX for five consecutive days.

Table 8: CRISPRi sgRNAs.

Target	sgRNA name	sgRNA (5' to 3')
HDAC4 exon 1	HDAC4-gRNA$_{238}$	CGCCGGAGCAGGGCTAGAGC
HDAC4 exon 1	HDAC4-gRNA$_{454}$	CGCGGGTGGAAAGGTCCAGA

3.3.7 Cell sorting and flow cytometric analysis

Transfected iXM001 iPSCs or differentiated cell clusters were dissociated with Accutase (Invitrogen). For sorting, cell suspension was filtered and resuspended in PSB with ROCK inhibitor (Sigma). Fluorescent Activated Cell Sorting (FACS) for Venus-expressing cells was performed on FACSAria I or II (BD Bioscience). Sorted iXM001 iPSCs were collected in E8 medium with ROCK inhibitor (Sigma) and seeded at low density to derive single-cell colonies. For flow-based analysis, dissociated cell clusters were fixed for 20 min at 4°C in cold BD fixation/permeabilization™ solution (BD Bioscience). Cells were washed twice in BD Perm/Wash™ Buffer (BD Bioscience) and incubated with primary antibodies in the dark for 2 hrs. at 4°C. Cells were washed 3x in BD Wash™ Buffer (BD Bioscience), resuspended in BD-FBS staining™ buffer (BD Bioscience) with secondary antibodies and incubated for 1 hr at RT. Primary and secondary antibodies are listed in table 9 and diluted 1:100. Stained cells

were washed 3x and analysis was performed on FACSAria II. FlowJo software was used to analyse data.

3.3.8 Glucose-stimulated insulin secretion (GSIS)

Human islets or human iPSC-derived β-like cells (about 10 clusters) were transferred into tubes and washed twice with Krebs-Ringer Bicarbonate buffer (KRB) containing 2.8 mM glucose. To allow equilibration, samples were incubated for 1 hr in low (2.8 mM) glucose containing KRB. Buffer was removed, replaced with fresh KRB containing 2.8 mM glucose and samples were incubation for 1 hr followed by an incubation in KRB containing 16.8 mM glucose. Samples were incubated for another hr. After both incubation periods the buffer was collected for human insulin specific ELISA analysis using an Ultrasensitive Insulin ELISA kit (Diagenics).

3.4 Histology

3.4.1 Immunofluorescence of cells

For immunofluorescence, Min6 cells and iPSCs were grown on glass coverslips. Cells were fixed for 20 min with 4% PFA at RT and washed three times with 1x PBS. Cells were blocked for 30 min in 0.1% Triton-PBS containing 3% donkey serum. Incubation with primary antibody was performed overnight at 4°C in blocking solution. The next day cells were washed three times for 10 min each in 0.1% Tween-PBS, incubated for 1 hr at RT in secondary antibody solution (in blocking buffer) and washed as above. Coverslips were mounted on slides using Dako mounting medium. The antibodies and dilutions are listed in table 9.

3.4.2 Embedding and cryosectioning of differentiated cell clusters

For histological analysis, cell clusters were fixed in 4% PFA for 15 min at RT. Following fixation, cell clusters were washed in PBS and equilibrated in 15% sucrose solution (PBS) at RT. Cell clusters were first embedded in 2.5% low-melting agarose, then in O.C.T. compound (Tissue-Tek®, Sakura®, Finetek) and subsequently frozen and stored at -80°C. Cryosections were cut at a thickness of 10 µm using a CM3050 S Leica cryostat and collected on standard glass slides (Thermo Scientific).

3.4.3 Immunofluorescence staining on cryosections

Before immunostaining, slides were incubated at 37°C for 45 min to remove agarose. The cluster sections were circled with a PAP pen to provide a hydrophobic barrier for application of solutions. Next, sections were blocked for 30 min at RT with 0.1% Triton-PBS containing 3% donkey serum. Primary antibodies were diluted according to table 9 in blocking buffer and sections were incubated overnight at 4°C in 500 µl primary antibody solution. The next day, slides were washed 3x for 5 min each in PBS with 0.1% Tween. Secondary antibodies and Hoechst were diluted 1:750 in blocking buffer. Slides were incubated for 40 min with secondary antibody conjugated to Alexa fluorophores in blocking buffer. Before imaging, slides were washed in PBS + 0.1% Tween and mounted in DAKO® fluorescence mounting medium. Imaged were acquired on a Zeiss LSM 700 confocal microscope using a 40x oil immersion objective.

Table 9: Antibodies and dyes used for immunofluorescence staining.

Antibody/Dye probe	Company, catalogue number	Host species	Dilution
Primary antibodies			
anti-c-Myc	MERCK, OP10	mouse	1:250
anti-C-peptide	Cell Signaling, 4593S	rabbit	1:250
anti-Gata6	Abcam, ab22600	rabbit	1:250
anti-Glucagon	Immunostar Inc., 20076	rabbit	1:250
anti-Insulin	Invitogen, PAI-26938	guinea-pig	1:250
anti-Mafa	Biomol (BETHYL), ICH-00352	rabbit	1:250
anti-Nkx6.1	Hybridoma Bank, F55A10	mouse	1:250
anti-Oct3/4	BD Transduction LabTM, 611203	mouse	1:100
anti-Pdx1	Abcam, ab47308	guinea-pig	1:500
anti-Somatostatin	Santa Cruz, sc-7819	goat	1:100
Secondary antibodies			
anti-guinea-pig IgG 647	Dianova (Jackson), 706-605-148	donkey	1:750
anti-goat IgG 594	Invitrogen, A11058	donkey	1:750
anti-mouse IgG 488	Invitrogen, A21202	donkey	1:750
anti-mouse IgG 594	Invitrogen, A21203	donkey	1:750
anti-rabbit IgG 488	Invitrogen, A21206	donkey	1:750
anti-rabbit IgG 594	Invitrogen, A21207	donkey	1:750
Hoechst 33342	Invitrogen, H1399	-	1:750

3.4.4 Whole-mount Immunofluorescence on differentiated cell clusters

iPSC-derived cell clusters were fixed in 4% PFA and stained as whole-mounts. Immunostaining was performed as described in section 3.4.3. Primary antibodies and Alexa-conjugated secondary antibodies are listed in table 9. However, secondary antibodies were used at a dilution of 1:500. Hoechst 33342 was used as nuclear counterstaining, and clusters were imaged in MaTek dishes using a Zeiss LSM 700 confocal microscope.

3.4.5 Alkaline phosphatase staining

For alkaline phosphatase staining iPSCs were fixed in 4% PFA for 15 min at RT and then stained with the NBT/BCIP alkaline phosphatase staining solution (Roche). After 1 to 2 hrs. the reaction was stopped with PBS and cells were imaged using an Axio Observer microscope.

3.5 Bioinformatic methods

3.5.1 Total RNA Seq and bioinformatic analysis

Total RNA concentration and RNA integrity of each sample were determined with NanoDrop and Qubit 4 Fluorometer (Thermo Fisher Scientific). RNA library preparations, sequencing reactions, and initial bioinformatics analysis were conducted by GENEWIZ. Briefly, data was generated with an Illumina HiSeq 2x150 PE HO configuration. Sequence reads were trimmed to remove adapter sequences and nucleotides with poor quality (Trimmomatic v.0.36). Using the STAR aligner v.2.5.2b the trimmed reads were mapped to the Homo sapiens GRCh38 reference genome available on ENSEMBL. Gene expression between distinct groups were compared using DESeq2.

3.5.2 Gene Ontology analysis

Gene Ontology analysis was performed using the publicly available David website (https://david.ncifcrf.gov.home.jsp).

3.6 Statistics

Statistical analyses were performed using GraphPad Prism. Unless stated otherwise, data are shown as mean ± standard error of the mean (SEM) and statistical significance ($p<0.05$) was determined using Student's T-test.

Table 10: Primers used for RT-qPCR in *Xenopus leavis* samples.

Target gene	Sequence (5' to 3')
insulin	ATGGCTCTATGGATGCAGTG
	AGAGAACATGTGCTGTGGGA
pdx1	GGGCTCTTGTGTAGGCTGTC
	CCTACAGCAACCCCTTGGTA
ptf1a	ATGGAAACGGTCCTGGAGCA
	GAGGATGAGAAGGAGAAGTTG
odc	TTCGGGTGATTCCTTGCCAC
	GCCATTGTGAAGACTCTCTCCAATC

Table 11: Primers used for RT-qPCR in mouse samples.

Target gene	Sequence (5' to 3')
Chga	CAAGTTTTTGCCCTTCCTGT
	CAGCGAGTCGGAGATGACTT
Ins2	CACCAGCCCTAAGTGATCCG
	GCCATGTTGAAACAATAACCTTCC
Isl1	GCGGCCTCTGCAAATGGCAG
	CTCCGGCTGCTTGTGGACGT
Neurod1	AAGGCAAGGTGTCCCGAGGC
	CAGCTGGTATCCATGTCCGT
Mafa	CCGCTTCTGTTTCAGTCGGA
	CATCAGCCCGCTCTCGCTGT
Nkx6.1	ACTTGGCAGGACCAGAGAG
	GCGTGCTTCTTTCTCCACTT
Pdx1	GGCGGGGCCGGGAGATGTATT
	CCACCAAAGCTCACGCGTGGA
Pcsk1	GGGTCAGATTGGATCACTTG
	CACGTCACACGATCATCATC
36B4	GGCCCTGCACTCTCGCTTTC
	TGCCAGGACGCGCTTGT

Table 12: Primers for RT-qPCR in human samples.

Target gene	Sequence (5' to 3')
OCT4	CTCTGCAGATTCTGACCGCA
	TCCTCTTCATGGGTGAGGGT
CD49a	TCCTCACTGTTGTTCTACGCTG
	ACGGAGAACCAATAAGCACC
FEV	TCTCTTCAAGGACGGGAAGAAC
	GAAACTGCCACAGCTGGATCT
FOXA2	TGCACTCGGCTTCCAGTATG
	CATGTTGCTCACGGAGGAGT
GAPDH	AGCTCACTGGCATGGCCTTC
	CGCCTGCTTCACCACCTTCT
GATA4	CCCAGACGTTCTCAGTCAGTG
	GCTGTTCCAAGAGTCCTGCT
GATA6	ACCACCTTATGGCGCAGAAA
	ATAGCAAGTGGTCTGGGCAC
GCG	GCAACGTTCCCTTCAAGACAC
	ACTGGTGAATGTGCCCTGTG
GCK	CTTCCCTCAGTTTTTCGGTGG
	TTGATTCCAGCGAGAAAGGTG
GLI1	GACTCCAGCCCTGGACCG
	GGTGGGGTCATCGAGTTGAA
GLI2	GCCTCTCCTTTGGTGGTGG
	CATGTCAATCGGTAGGGGCG
HDAC4	CCTCTTCTGCAGCACATGGT
	GACTGTGCGTGGAGGGG
HES1	TCAACACGACACCGGATAAAC
	GCCGCGAGCTATCTTTCTTCA
HNF1B	TCTCAACAAGGGCACCCCTA
	GCAGCTGATCCTGACTGCTTT
PTF1a	GTCATCATCTGCCATCGGG
	CTAGGGGAGGGAGGCCATAA
INS	GCAGCCTTTGTGAACCAACAC
	CCCCGCACACTAGGTAGAGA
ISL1	ACATGCTTTGTTAGGGATGGGA
	TCGTTCTTGCTGAAGCCGAT
KIR6.2	TGTGTCACCAGCATCCACTC
	CACTTGGACCTCAATGGAGAA
MAFA	GGCTTCAGCAAGGAGGAGGT
	TGGCACTTCTCGCTCTCCAG
MAFB	CCCGACCGAACAGAAGACA
	ACTGGGTGCGAGCCGATGAG

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NANOG	TCCAACATCCTGAACCTCAGC
	CAGGCATCCCTGCGTCAC
NEUROD1	GACACGAGGAATTCGCCCAC
	CCCACTCTCGCTGTACGATTT
NGN3	TTTTCTCCTTTGGGGCTGGG
	CTCACGGGTCACTTGGACAG
NKX2.2	TTCCAGAACCACCGCTACAAG
	GGGCGTCACCTCCATACCT
NKX6.1	TGGCCTATTCGTTGGGGATG
	TGTCTCCGAGTCCTGCTTCT
PAX4	AAATGGCGTCGGCAAGAGAA
	AGATGATTCCTGGGGCAACC
PAX6	AGGATGTTGAACGGGCAGAC
	TCTCCCCCTCCTTCCTGTTG
PCSK1	GCTAAATGCCAAAGCTCTGGTGG
	GCTTTCAGGGCTCTGGGCTC
PDX1	CACATCCCTGCCCTCCTAC
	GAAGAGCCGGCTTCTCTAAAC
PTCH1	GAAGAAGGTGCTAATGTCCTGAC
	GTCCCAGACTGTAATTTCGCC
SMO	GAAGTGCCCTTGGTTCGGA
	GCAGGGTAGCGATTCGAGTT
SOX17	TTCGTGTGCAAGCCTGAGAT
	TAATATACCGCGGAGCTGGC
SOX2	ATGACCAGCTCGCAGACCTACA
	GGACTTGACCACCGAACCCA
SOX9	GGAGACTTCTGAACGAGAGCG
	GAGATGTGCGTCTGCTCCG
SST	CCACTCTCCAGCTCGGCTTT
	TTGGCCAGTTCCTGCTTCCC
SUR1	AGACCCTCATGAACCGACAG
	GGCTCTGTGGCTTTTCTCTC

4 RESULTS

4.1 Generating a priority lists for studying putative disease-associated genes for diabetes

4.1.1 Screening of variants combines genetic information with molecular studies

The research group of PD Dr. Raile at the Charité/Experimental and Clinical Research Center (ECRC) established a unique cohort of families with puberty-onset diabetes of unknown cause. All individuals (n = 94) developed diabetes before the age of 18 years, tested negative for β-cell autoantibodies and were subjected to next-generation sequencing (whole exome and whole genome). Suspected pathogenic variants were verified by Sanger sequencing and patients with mutations in known monogenic diabetes genes (e.g. *GCK*, *KCNJ11* and *HNF1β*) were excluded from the cohort.

Next-generation sequencing in more than 35 families led to the identification of unique heterozygous mutant variants in genes coding for HDAC4, GLI1, GLI2, Dedicator of cytokinesis protein 1 (DOCK1), Dynamin-1-like protein (DNM1L), and others (K. Raile, Charité, Berlin, partially published data) [148]. So far, mutations in these genes have not been associated with diabetes in publicly available databases. From the initial list of these putative new candidate disease-genes for diabetes, *HDAC4*, *GLI1* and *GLI2*, have been retained for further functional studies (Fig. 6). Specifically, I focused on gene candidates, which are abundantly expressed in mouse pancreatic progenitor cells and have a putative role in transcriptional networks during embryogenesis [198]. Based on these criteria, two variants in *HDAC4* (c.A680G, c.G700A), two variants in *GLI1* (c.C119G, c.G1414T) and four different variants in *GLI2* (c.C1859T, c.G3099C, c.G4145A, c.C4661T) were selected (Table 13).

Genetic evidence that variants in these genes might cause diabetes arises from four (*HDAC4*), two (*GLI1*), or five (*GLI2*) independent families, respectively (Table 13). The majority of the heterozygous de novo variants were predicted to be pathogenic by at least one *in silico* predictive algorithm (see section 4.1.2). Based on preliminary data and functional prediction, the appropriate model system for primary characterization of the variants was chosen. These initial investigations allowed me to identify those that are more likely of interest for pancreas development and β-cell differentiation. Ultimately, one variant per gene candidate was selected to be studied in human iPSC-based models of β-cell development.

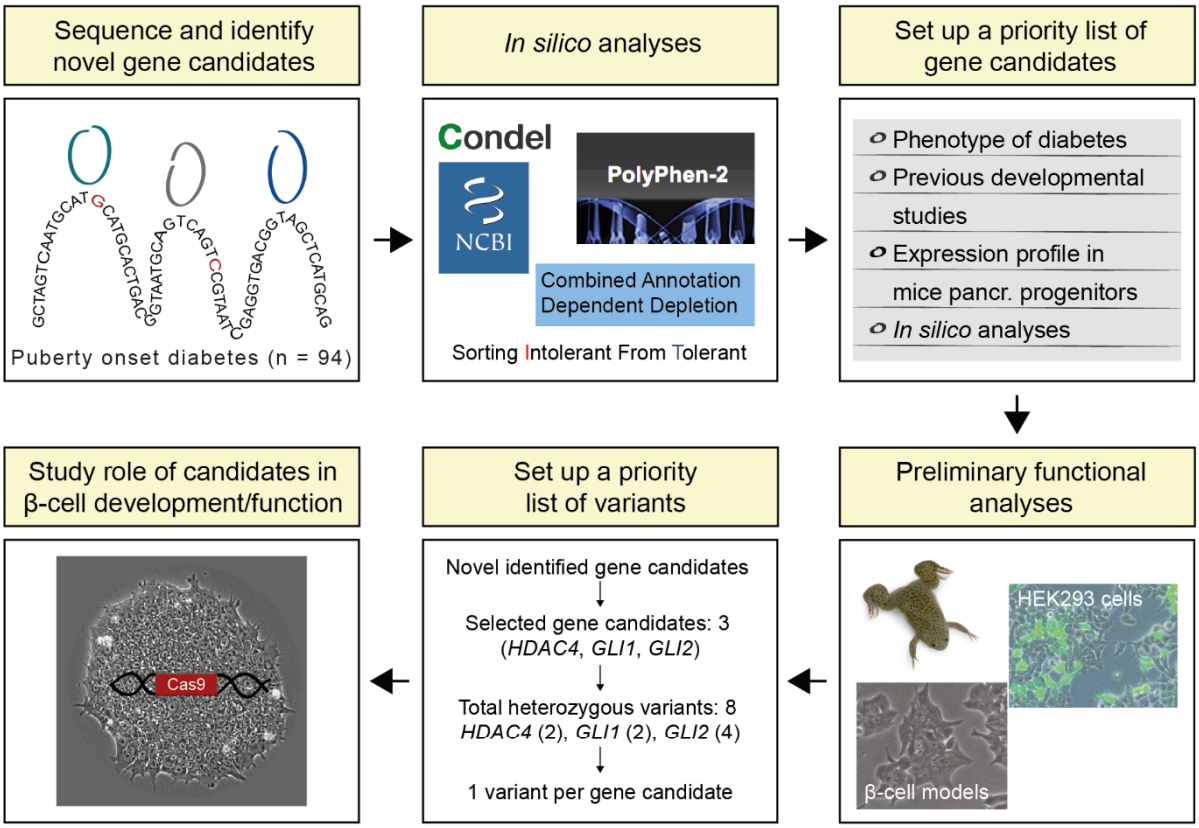

Fig. 6: Schematic of the study of putative new candidate disease-genes for diabetes in β-cell development and function. Next-generation sequencing (exome and whole genome) of a cohort of individuals (n = 94) with puberty-onset diabetes of unknown pathogenesis led to the identification of unique mutant variants in genes coding for HDAC4, GLI1 and GLI2. These gene candidates were prioritized for functional analysis and studied in human iPSC-based models for β-cell development. Pancreatic (Pancr.).

Thus, I set up a working pipeline to prioritize putative pathogenic variants for in-depth characterization in human iPSC models by combining genetic information with molecular studies in various *in vivo* and *in vitro* models for pancreas development, β-cell differentiation and function (Fig. 6). The results of each step from bioinformatics, preliminary functional analysis to finally iPSC disease modelling are described in the subsequent sections.

4.1.2 *In silico* analysis of identified putative disease-causing variants

The rapid progress of advanced genomic technologies has provided new opportunities to identify novel mutations in monogenic diabetes patients. Since most mutations causing monogenic diabetes are missense mutations resulting in the change of a single amino acid residue [375]. It is very challenging to distinguish them from a large number of missense variants without causative role in diabetes. Consequently, various *in silico* prediction algorithms, including SIFT (Sorting Intolerant From Tolerant), Polyphen-2 (Polymorphism Phenotyping v2), Provean, CADD (Combined Annotation Dependent Depletion) and Condel have been developed to help in assessing the pathogenicity of missense mutations. These

and other bioinformatic tools are very useful to estimate the potential pathogenicity of a variant and establish a framework for further investigations at the molecular level.

To determine the functional impact of the identified missense variants, I analysed all variants in *HDAC4*, *GLI1* and *GLI2 in silico* using SIFT, Provean, PolyPhen-2, Condel and CADD [376–382]. The results are summarized in table 13. With the exception of variant c. A680G in *HDAC4* and the variant c.C1859T in *GLI2*, all identified variants were predicted to be deleterious (SIFT, Provean, and/or Condel) and damaging (PolyPhen-2) by at least one *in silico* predictive algorithm and/or had a CADD score (PHRED-like) above 20. CADD integrates several annotations into a single score and a PHRED-like score of at least 20 indicates that the tested variant is among the top 1% of deleterious variants in the human genome. Across the eight variants, the variant c.C4661T (or p.P1554L) in *GLI2* had the highest CADD score and was predicted to be pathogenic by all applied bioinformatics tools, suggesting that this variant is likely biologically relevant. Furthermore, I-mutant 2.0 was used to predict changes in the stability upon mutation, by mapping the different variants to the native protein structure [383]. Here I noted that 99% of the reported missense variants were estimated to reduce protein stability.

Lastly, I analyzed the position of each variant in the respective protein sequence, since mutations inside or close to critical protein domains or essential residues might impair protein function. For instance, the two identified variants in HDAC4 (p.H227R and p.D234N) localize within the conserved binding site for the TF myocyte enhancer factor 2 (MEF2) (Fig. 7A). Moreover, the residues p.H227 and p.D234 are close to post-translational phosphorylation sites (e.g. Ser210 and Ser246) (Fig. 7A). These were shown to mediate the interaction between HDACs and the 14-3-3 family of proteins, which are known to bind specifically to conserved phosphoserine-containing motifs [384–386]. Changes in these phosphorylation sites have been suggested to negatively regulate HDAC4 by preventing its nuclear localization [384]. Therefore, the N-terminal region of the HDAC4 protein, in which the detected variants lie, can be considered as an important regulatory HDAC4 domain.

In the case of GLI1, both variants (p.P168R and p.G600C) are not in close proximity to essential protein domains, such as the DNA binding zinc finger domains or Suppressor of fused homolog (SUFU) interaction site (Fig. 7B). However, the amino acid residue changes from hydrophobic to a charged (P168R) or polar (G600C) residue, respectively. This could have an adverse effect on protein folding and thus protein function. Moreover, unlike other components of the SHH signaling pathway, such as PTCH1, disease-linked mutations in *GLI1* are very rare [387,388]. So far only two missense mutations are listed in the human gene mutation database (HGMD) associated with bicuspid aortic valve or inflammatory bowel disease [389–392].

Table 13: Overview of identified variants in cohort of families with rare diabetes types.

Gene	No. of families	Exon	c.Position	p.Position	Phenotype* and family history	MAF (ExAC database)	SIFT[1]	PROVEAN[2]	PolyPhen2[3]	CADD PHRED[4]	Condel[5]	I-Mutant[6]
HDAC4	4	7	A680G	H227R	T1D/14yrs/Ins	rs148880349 (76/121340)	Tolerated	Neutral	Benign	21.8	Neutral	Decreases
		7	G700A	D234N	T1D/8yrs/Ins	rs549858743 (5/121326)	Tolerated	Deleterious	Benign	27.9	Neutral	Decreases
GLI1	2	3	C119G	P168R	MODYX/13yrs/Ins Mother & grandmother T2D	rs542007910 (246/120346)	Deleterious	Neutral	Benign	21.9	Deleterious	Decreases
		11	G1414T	G600C	T2D/14 yrs/Ins Mental retardation	rs147997893 (3/6500)	Tolerated	Neutral	Pos. damaging	22.9	Deleterious	Decreases
GLI2	5	12	C1859T	T620M	T2D/16 yrs/OADS	rs1427r75128 (23/120942)	Tolerated	Neutral	Benign	13.21	Neutral	Increases
		14	G3099C	E1033D	MODYX/7 yrs/Ins	Not in dbSNP (2/79806)	Deleterious	Deleterious	Prob. damaging	24.4	Deleterious	Decreases
		14	G4145A	R1382H	T2D/16 yrs/OADS	rs200080112 (19/101796)	Deleterious	Deleterious	Prob. damaging	25.2	Deleterious	Decreases
		14	C4661T	P1554L	T1D/5 yrs/Ins Father diabetes	Not in dbSNP (4/121120)	Deleterious	Deleterious	Prob. damaging	28.4	Deleterious	Decreases

* Treatment: insulin (ins), oral antidiabetic drugs (OADs)
T1D: non-immune, insulin-dependent diabetes, sporadic, non-familial cases. MODYX: familial diabetes, dominantly inherited, no mutation in established MODY-genes found. Prediction websites: [1]https://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html; [2]http://provean.jcvi.org/index.php; [3]http://genetics.bwh.harvard.edu/pph2/; [4] https://cadd.gs.washington.edu/; [5]https://bbglab.irbbarcelona.org/fannsdb/help/condel.html; [6]http://folding.biofold.org/i-mutant/i-mutant2.0.html. Years (yrs.), possibly (pos.), probably (prob.).

Mueller LM, Raile R, Spagnoli FM (unpublished)

One variant in GLI2 (p.T620M) is next to the last DNA binding zinc finger domain (Fig. 7C). Furthermore, the other three C-terminal variants (p.E1033D, p.R1382H and p.P1554L) are within the transactivation domain, whose deletion was shown to inhibit GLI2 specific transcriptional activity (Fig. 7C) [393,394]. To be noted, several heterozygous *GLI2* mutations have been reported to cause forebrain and pituitary defects [395–397]. Nevertheless, these symptoms were not present in any of the sequenced patients. Mutations in *GLI1* and *GLI2* were of specific interest because previous studies underscored the significance of the HH signaling pathway for pancreas organogenesis, differentiation of pancreatic progenitors, β-cell expansion and glucose metabolism (see chapter 1.1.5.2).

It is now increasingly evident that under physiological conditions a large number of human proteins lack fixed, ordered 3D structures as a whole or have regions that are less likely to form a defined conformation. These proteins and regions are referred to as intrinsically disordered proteins or regions, respectively. Nevertheless, these flexible structures are highly dynamic and involved in various crucial biological functions [398–400]. Importantly, many proteins with disordered regions have been associated with diseases, such as diabetes, and alterations in these regions may increase disease risk or even cause a disease. Therefore, I used the Protein DisOrder prediction System (PrDOS) to estimate natively disordered regions of the protein chain from its amino acid sequence [401]. Interestingly, while the conserved regions of the proteins, like the deacetylase domain of HDAC4 or the zinc finger domains of the GLI proteins mapped to ordered regions, the variants affected amino acids residing in disordered regions (disorder probability > 0.5) (Fig. 7). Moreover, the amino acid substitution at GLI2 p.P1554 position decreased the disorder probability.

In conclusion, the *in silico* analyses of the different identified variants in our unique cohort of diabetic patients highlighted their potential adverse effect and supported the rational for further investigations to study the underlying disease mechanism. To confirm these results and further prioritize the different variants, I conducted preliminary functional studies in distinct *in vivo* and *in vitro* models.

4.1.3 Studies to characterize the role of *HDAC4* during pancreas development

Mutations in HDAC4 have not yet been associated with embryonic pancreas development or β-cell fate specification. I started by studying the function of wild-type (WT) and mutant human variants of *HDAC4* in gain-of-function experiments in *Xenopus laevis* embryos and mouse Min6 insulinoma cells, a well-established β-cell line model [402,403]. The *Xenopus* is a remarkable model system to study early events in development, which are conserved across vertebrate species [71,404,405]. Apart from its close evolutionary

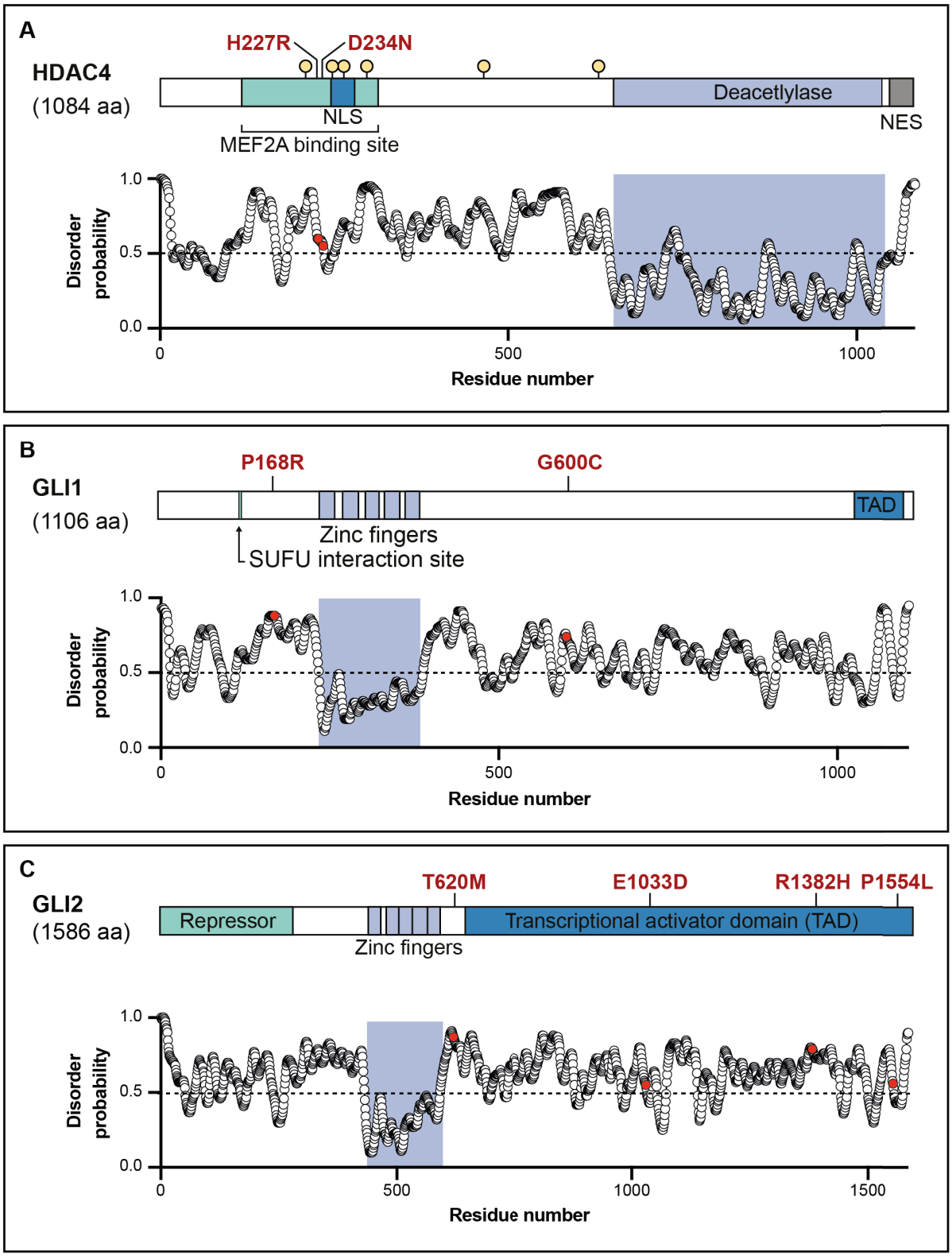

Fig. 7: Protein structure and disordered regions of HDAC4, GLI1 and GLI2. (**A**, **B**, **C**) Schematic representation of identified putative disease-associated variants in HDAC4, GLI1 and GLI2, respectively. Several variants are in/close proximity to essential protein domains or important residues. Protein domains, like the deacetylase domain (HDAC4), zinc finger domain (GLI), transcriptional activator domain (GLI), repressor domain (GLI2) and crucial protein binding sites are shown in colored boxes. Phosphorylation sites in HDAC4 are shown as yellow dots. Variants are labeled in red. Disorder probability plots are based on predictions from PrDOS. A disorder probability above 0.5 is considered as disordered. Transcriptional activator domain (TAD), Myocyte Enhancer Factor 2A (MEF2A), Suppressor of fused homolog (SUFU).

relationship to higher vertebrates, the *Xenopus* offers the following incomparable advantages over mammalian models: (i) high fecundity and external development, (ii) large embryos suitable for surgical manipulations and (iii) rapid functional genomics. These features together with the conservation of the mechanisms underlying pancreas development render the *Xenopus* an ideal system to functionally characterize novel identified gene candidates and test the causality of putative pathogenic variants [139,406].

4.1.3.1 Ectopic expression of HDAC4 in Xenopus laevis embryos alters insulin expression

First attempts to study the putative mechanism by which HDAC4 and the identified mutant variants contribute to the development of diabetes were performed by gain-of-function experiments in *Xenopus laevis* embryos. The dorsal and ventral pancreatic buds are apparent by NF stage 37/38 and located at the dorsal endodermal midline or close to the prospective hepatic bud, respectively. NF refers to the specific stage of *Xenopus* development described by Nieuwkoop and Faber, 1967. By NF stage 40 the dorsal and two ventral buds will have fused to subsequently give rise to the mature pancreas [406]. Previous fate mapping experiments have shown that during gastrulation the prospective pancreatic endoderm forms in the dorsal-vegetal quarter of the frog embryo around the dorsal blastopore lip. Therefore, these endoderm cells can be specifically targeted for microinjection and gene manipulation during early development. Moreover, it has been shown that dorsal endoderm explants isolated by dissection from gastrula stage embryos and cultured in isolation express pancreatic genes, while ventral endoderm fail to do so [407,408]. Thanks to this early regionalization of the endoderm, I have established a robust assay to study the effect of ectopic expression of WT and mutant *HDAC4* on pancreas formation by qRT-PCR and whole-mount *in situ* hybridization (Fig. 8A).

First, the ORF of human *HDAC4* WT or mutant was cloned into the pCS2^{++} expression vector. The generated plasmids were linearized and used as template for *in vitro* transcription of mRNAs. After purification and validation, the obtained mRNA was injected into both ventral and dorsal endoderm of the developing embryo. Both endoderm regions were dissected and cultured until tadpole stage, when pancreatic genes start to be expressed, and then examined by RT-qPCR. Independently from the injected mRNA the embryos developed in a manner grossly indistinguishable from un-injected controls. Furthermore, no induction of pancreatic genes was observed in ventral-vegetal injected and derived explants, which are normally devoid of pancreatic markers. Thus, *HDAC4* appears not to be sufficient to promote ectopic pancreatic cell fates. In line with this, overexpression of neither human *HDAC4* WT nor *HDAC4*

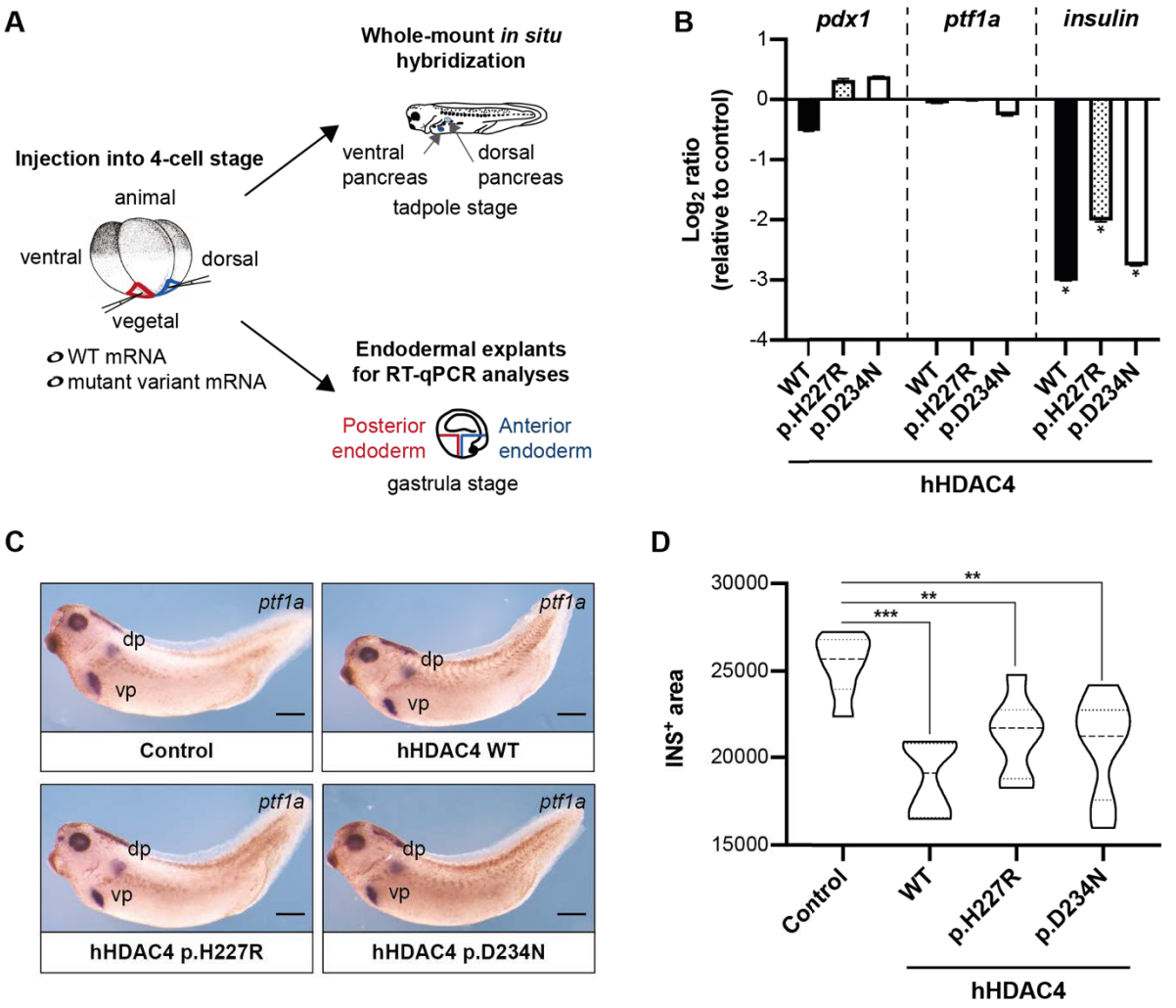

Fig. 8: Investigate the role of HDAC4 during pancreas development *in vivo*. (**A**) Examples illustrating different types of assays that are used in *Xenopus* embryos to study pancreatic phenotypes. (**B**) RT-qPCR analysis of HDAC4 WT and mutant variants (p.H227R and p.D234N) of HDAC4 in vegetal-dorsal (VD) injected explants. HDAC4-mRNA (1 ng) was injected into two VD blastomeres of 4-cell stage *Xenopus* embryos, VD explants were dissected at gastrula stage and assayed at tadpole stage for the indicated pancreatic genes by RT-qPCR. Data were normalized to ornithine decarboxylase and represented as log_2-fold changes relative to un-injected control samples. Error bars represent SD. *p < 0.05 (**C**) Whole-mount *in situ* hybridization analysis of *ptf1a* in un-injected control, HDAC4 WT, HDAC4 p.H227R and HDAC4 p.D234N injected embryos, respectively. Scale bar, µm 500. (**D**) Violin plot of insulin positive area measured in whole-mount *in situ* embryos un-injected or injected VD with HDAC4 WT, HDAC4 p.H227R or HDAC4 pD234N, respectively. **p < 0.01, ***p < 0.001.

mutant variants in dorsal endoderm significantly affected the expression level of *pdx1* and *ptf1a*, which are essential for pancreas fate specification (Fig. 8B) [70,206]. *In situ* hybridization of injected *Xenopus* embryos supported this observation, showing no change in *ptf1a* expression in the dorsal or ventral pancreatic bud (Fig. 8C). However, preliminary data showed reduced insulin expression in embryos overexpressing human *HDAC4* (Fig. 8B). Consistently, whole-mount *in situ* hybridization showed a reduction in the area of the insulin positive cells in human *HDAC4*-injected *Xenopus* embryos (Fig 8D).

In summary, these preliminary studies suggested that deregulation of *HDAC4* impairs β-cell development as manifested by the reduced insulin expression compared to un-injected controls. However, the results were inconclusive regarding the effect of the identified *HDAC4* variants, therefore I further investigated their potential role in maintaining the function of pancreatic β-cells in mouse Min6 cells.

4.1.3.2 HDAC4 p.D234N impairs maintenance of β-cell identity in mouse Min6 cells

The murine pancreatic β-cell line (Min6) was established from insulinomas obtained by targeted expression of the simian virus 40 T-antigen gene in transgenic mice [403,409]. This cell line has morphological characteristics of pancreatic β-cells and secretes insulin upon glucose stimulation. Thus, Min6 cells are comparable to normal islets. Hence, it is a good model to study β-cell function and glucose-dependent insulin secretion, as well as, membrane and cytosolic ion physiology upon overexpression of WT and mutant gene variants [402,410,411].

After transfecting Min6 cells with plasmids harbouring the WT or patient-specific mutant variant of *HDAC4*, I characterized the expression of various β-cell critical genes by RT-qPCR. Cells transfected with HDAC4 p.D234N displayed decreased levels of insulin (Fig. 9). Furthermore, overexpression of HDAC4 p.D234N led to downregulation of key β-cell genes, like *Pdx1*, *Isl1*, *Psck1*, one of the prohormone convertases that catalyses (pro)insulin processing, and *Cgha* (Fig. 9). These genes are crucial for the function of mature β-cells and

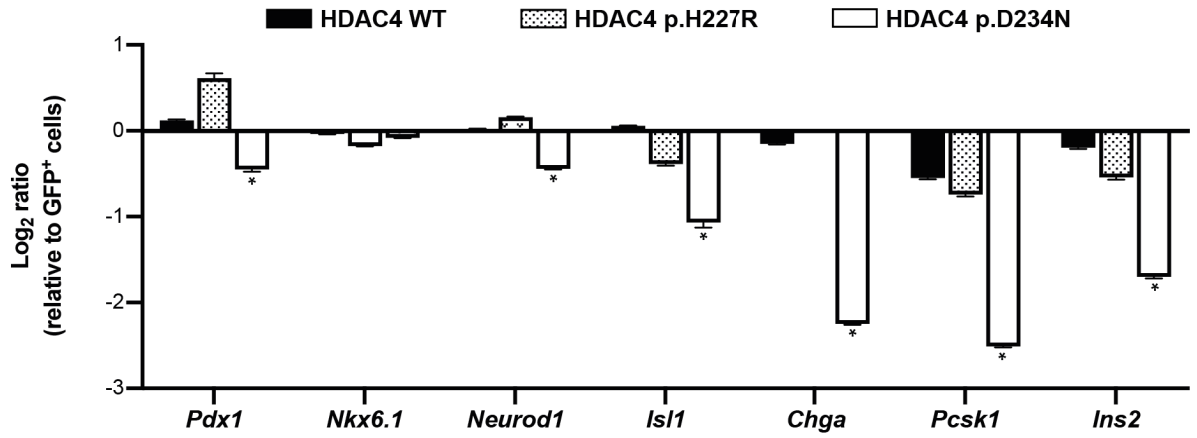

Fig. 9: *In vitro* characterization of HDAC4 WT and patient variants. RT-qPCR analysis of Min6 cells transfected with HDAC4 WT and mutant variants (p.H227R and p.D234N) for the indicated pancreatic genes. Data were normalized to acidic ribosomal phosphoprotein P0 (*36B4*) and represented as log_2-fold changes compared to GFP-transfected cells. Error bars represent SD. *p < 0.05.

impaired expression is linked to loss of β-cell identity [75,412,413]. Instead, transfection of Min6 cells with WT and HDAC4 p.H227R had no effect on the expression of these genes.

These results together with the ectopic expression of human *HDAC4* mutant variants in *Xenopus* embryos suggest that *HDAC4* might play a role in maintaining β-cell identity.

4.1.4 The diabetic patient variants in *GLI1* and variant c.C4661T in *GLI2* impair activation of HH signalling pathway

Previous studies have demonstrated that GLI proteins play a role in pancreatic development either acting as transcriptional repressor or activator of the HH signalling pathway. During pancreas organogenesis, repression of HH signalling is required for pancreas fate specification and cell differentiation. However, for the maintenance of endocrine function, active HH signalling is essential. Conflicting evidence also suggests that high HH levels in insulin-producing cells impairs β-cell function by interfering with the mature β-cell differentiation state. Thus, a precise spatiotemporal HH regulation appears to be important during pancreas development and in adult β-cells.

To test if the novel identified variants in the human *GLI1* and *GLI2* genes interfere with their transcriptional activity, I performed a GLI-mediated transcriptional activation assay using a GLI-responsive luciferase reporter. In detail, I co-transfected HEK293T cells with plasmids expressing WT or mutant GLI1/GLI2 together with a GLI-responsive firefly luciferase reporter construct and a constitutive renilla luciferase reporter (Fig. 10A). The measured firefly luciferase activity was normalized to the renilla luciferase activity. Both point mutations in *GLI1*, which are located outside of the activator domain, diminished significantly the GLI-induced luciferase activity of the reporter. In comparison to *GLI1 WT*-transfected cells the activity was reduced by approximately 30% (Fig. 10B). A similar result was observed for the variant c.C4661T, which lies within the activator domain of GLI2 (Fig. 10C). The impaired transcriptional activation potential of these three GLI variants was consistent and robust even under HH ligand stimulating conditions (e.g. use of SHH conditioned medium). For the other three *GLI2* variants (c.C1859T, c.G3099C and c.G4145A) no significant reduction in activation could be determined across the biological triplicates. As expected, transfection with a mutated version of the GLI-luciferase reporter construct or replacing GLI1/GLI2 WT with GFP failed to induce GLI-dependent gene transcription (Fig. 10B and C).

In summary, these findings suggest that both *GLI1* variants, as well as the c.C4661T variant in *GLI2*, might be loss-of-function mutations, leading to deregulation of HH signalling thereby potentially contributing to the development of diabetes.

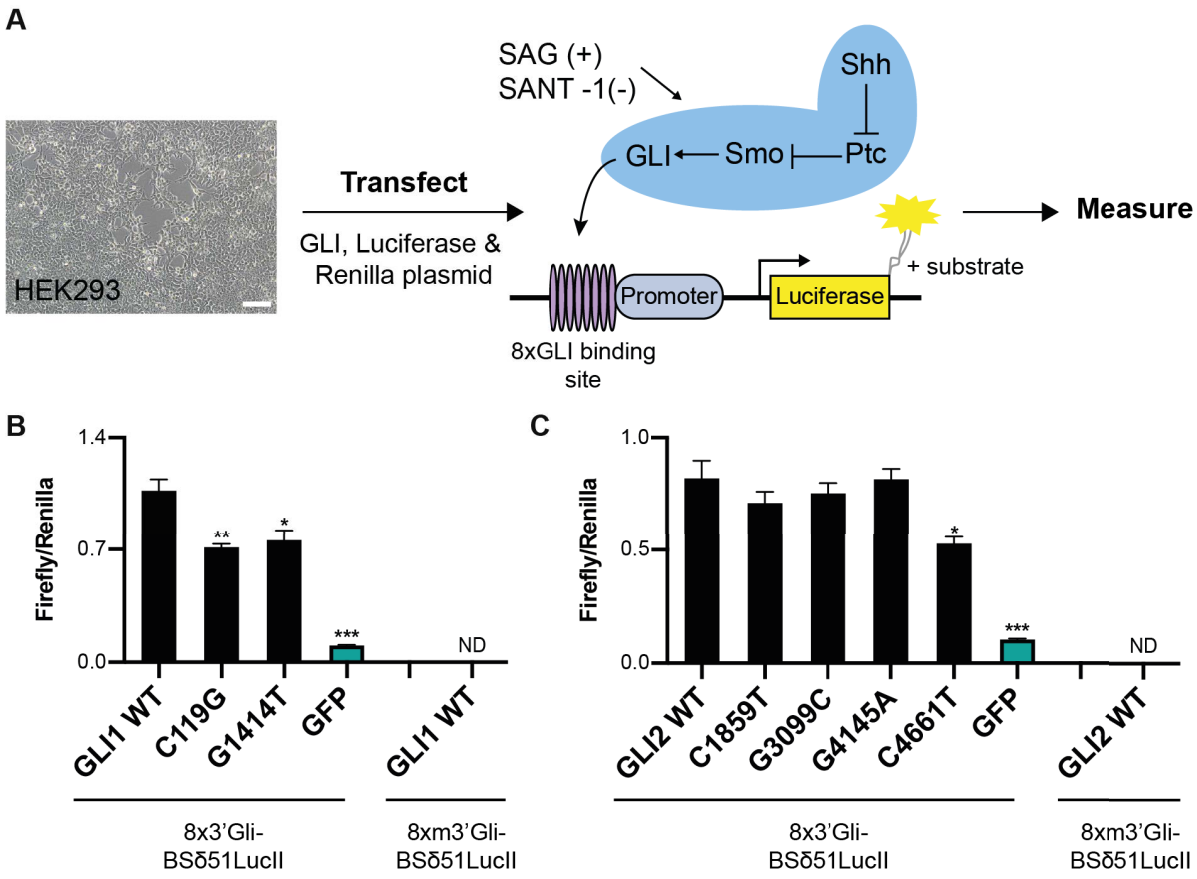

Fig. 10 Putative pathogenic variants in *GLI* genes impair transcriptional activation of the HH signalling pathway. (**A**) Schematic illustration of the HH pathway and GLI-dependent luciferase reporter assay. The WT reporter system (8x3'Gli-BSδ51LucII) carriers eight copies of Gli1-binding sites. Binding of GLI1 or GLI2 drives the expression of the luciferase reporter gene. However, if the binding sites of the luciferase reporter construct are mutated (8xm3'Gli-BSδ51LucII) the luciferase expression is not induced. Luciferase activities can be measured by luminescence. Treatment with SHH, SANT-1 (Smo antagonist) or SAG (Smo agonist) alter the HH pathway and thus luciferase activity. (**B**, **C**) GLI-luciferase assay to test transcriptional activation potential of putative pathogenic variants in GLI1 and GLI2, respectively. The WT or mutated luciferase reporter plasmid was co-transfected with an internal control plasmid (Renilla) and a DNA expression vectors as indicated. Results were normalized for transfection efficiency using Renilla luciferase and are represented as Firefly/Renilla activity ratio. The experiment was performed in triplicates and repeated independently three times. Error bars represent SD. *p < 0.05, **p < 0.01, ***p < 0.001. ND: not determined.

To further examine the effect of the GLI2 c.C4661T variant on the expression of β-cell markers, I transiently transfected Min6 cells with plasmids expressing a c-Myc tagged GLI2 WT or mutant variant. Interestingly, in GLI2 c.C4661T-transfected cells I observed a negative correlation between the expression of the patient GLI2 variant and MAFA. Specifically, in comparison to GLI2 WT-transfected cells, expression of *MafA* was strongly reduced in GLI2 c.C4661T-positive cells (Fig. 11A and B). This was confirmed by RT-qPCR in transfected bulk populations (Fig. 11C). The maturation factor MAFA is critical for the homeostasis of mature β-cells. Loss of MAFA is associated with altered β-cell identity and de-differentiation of β-cells, which is implicated in diabetes pathogenesis. Moreover, expression of *NeuroD1* was

significantly decreased in GLI2 c.C4661T transfected cells whereas the expression of *Pdx1*, *Pcsk1*, *Nkx6.1* and *Insulin* was not altered in GLI2 mutant transfected Min6 cells (Fig. 11C). Together, these results suggest a role of GLI2 in the maintenance of β-cell identity, which is disrupted by the putative pathogenic sequence variant c.C4661T.

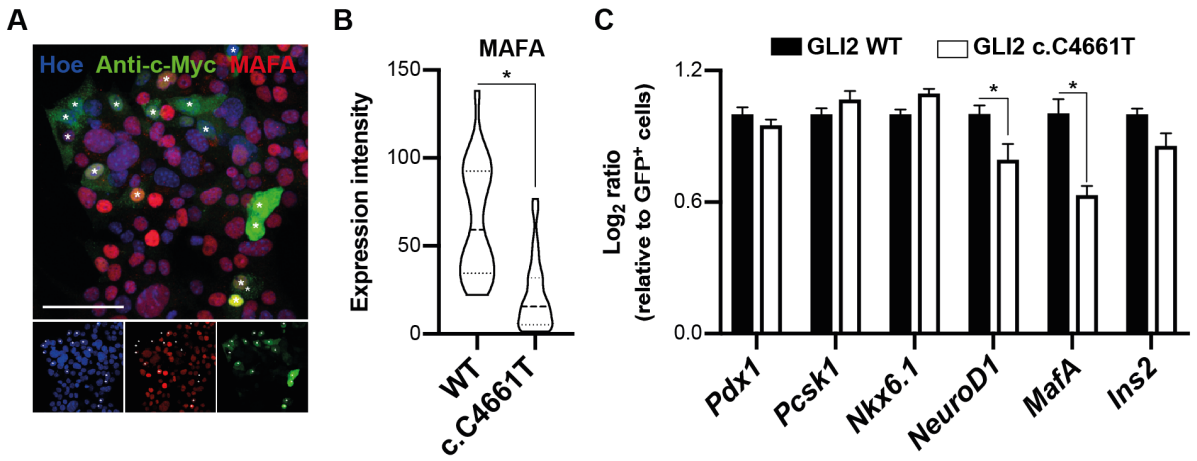

Fig. 11. GLI2 c.C4661T impairs *NeuroD1* and *MafA* expression in Min6 cells. (**A**) IF of MafA in GLI2 c.C4661T transfected Min6 cells. Scale bar, 100 µm. c-Myc tagged GLI2 c.C4661T transfected cells indicated by asterisk. (**B**) Relative expression intensity of MAFA in GLI2 c.C4661T transfected Min6 cells. Expression intensity of MAFA was normalized to intensity of Anti-c-Myc. (**C**) RT-qPCR of β-cell markers in GLI2 WT and GLI2 c.C4661T transfected Min6 cells. Values are normalized to 36B4 and relative to GFP. Representative RT-qPCR of n = 3. *p < 0.05.

In conclusion, the *in silico* analyses of the different mutant variants and the preliminary functional validation shed some light on the potential adverse effect of the gene candidate variants and support the rationale for further investigations. Based on these observations and the available patient information, I assigned priority to the patient variants *HDAC4* c.G700A, *GLI1* c.C119G and *GLI2* c.C4661T to generate an iPSC-derived β-cell disease model.

4.2 Establishing an iPSC platform to study novel intrinsic and extrinsic factors

Advances in iPSC technology have opened up the possibility of generating patient-specific stem cell lines to model diseases *in vitro*. Directed differentiation of iPSCs toward the pancreatic lineage is now a viable and attractive method to reveal the underlying molecular mechanism of mutations in patients with monogenic diabetes. To date, some iPSC lines have been established from diabetic and healthy donors and differentiated into insulin-secreting β-like cells following different approaches. Moreover, recent progress has been made to improve the differentiation of human embryonic stem cells into monohormonal β-like cells. Nevertheless, the generation of mature β-cells *in vitro* is facing several challenges, above all the variability in biological properties among individual iPSC lines. Thus, it is essential to set up an efficient, robust and scalable iPSC differentiation platform before studying disease-causing mutations or testing novel extrinsic factors.

4.2.1 Distinct iPSC lines can differentiate into β-like cells with varying efficiency

To establish a differentiation model system, I selected two iPSC lines, namely BiH005-A and iXM001, and differentiated them into β-like cells based on the previously published protocol by Russ et al. (Fig. 13A). The human BiH005-A iPSC line was derived by Dr. Sebastian Diecke (Pluripotent Stem Cell Core, MDC) from fibroblasts of a male (Asian ethnic origin) individual through induction of the four Yamanaka factors [305,414]. The original human XM001 iPSC line (hPSC^{reg} name: HMGUi001-A) was established by the group of Dr. Heiko Lickert (Helmholtz Center Munich) by reprogramming fibroblasts from a healthy female Caucasian donor [415]. Instead, I used the modified human iXM001 iPSC line provided by Dr. Ralf Kühn (Transgenics, MDC), which carries a doxycycline (DOX)-inducible Cas9 expression cassette inserted into the AAVS1 locus allowing the *de novo* introduction of mutations into an otherwise healthy iPSC line (Fig. 12) [331].

Before starting differentiation, the human BiH005-A and iXM001 iPSC line demonstrated the molecular hallmarks of pluripotent cells, including morphological features, normal karyotype and expression of pluripotency markers (see hPSC^{reg}) [416]. Prior to exposing BiH005-A and iXM001 iPSCs to the five-stage differentiation procedure, I induced sphere formation on an orbital shaker (Fig. 13B). Indeed, all current protocols for inducing pancreatic fate differentiation are based on 3D cultures. BiH005-A and iXM001 iPSCs readily formed cell clusters by self-aggregation with a diameter of 120-190 µm (Fig. 13B) Independently of the cell line used, clusters displayed high uniformity and lacked typical signs

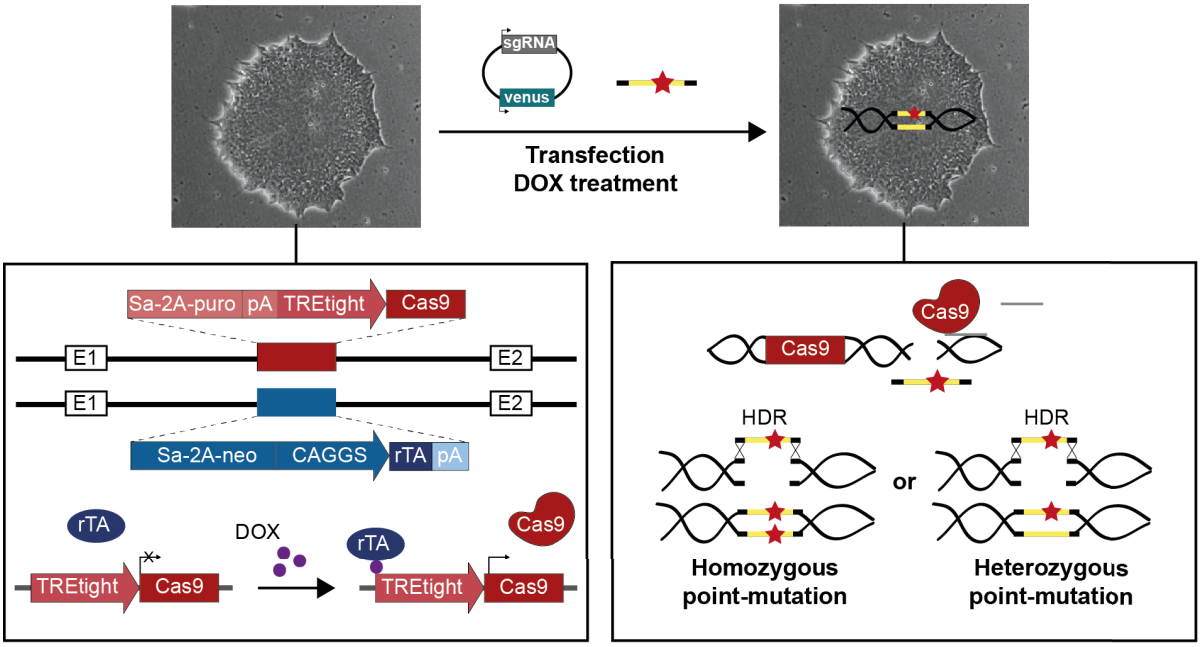

Fig. 12: Generation of patient-like iXM001 iPSC lines. DOX dependent expression of Cas9 is provided by a modified AAVS1 locus. The DOX-inducible promoter TREtight and the Cas9 gene are knocked in between the first two exons of the PPP1R12C gene (AAVS1 locus) on one allele. The other allele harbours the Transactivator gene rtTA3 expressed under the constitutively active CAGGS promoter. Addition of DOX mediates binding of rtTA to TREtight, thus activating the expression of Cas9. After transfection of iPSCs with a plasmid carrying the sgRNA together with a fluorescent reporter (venus) and a repair template (single stranded oligo-dinucleotide [ssODN]), precise knock-in alleles are introduced by HDR upon DOX-induction of Cas9. Adapted from Yumlu et al. (2017).

for spontaneous differentiation, suggesting that both lines are suitable for a 3D suspension-based differentiation approach (day 2, Fig. 13B). Subsequently, the cell clusters underwent differentiation, through successive intermediates, including endoderm (day 5), foregut (day 7), pancreatic progenitor (day 9), endocrine progenitor (day 14), and β-like cell (day 19) stage upon exposure to a well-defined set of cytokines and small molecules (Fig. 13A). At the foregut stage, bud-like clusters were detectable (day 7, Fig. 13B). For the iXM001 cell line differentiation, budding was already observed at the end of the endoderm stage (day 5, Fig. 13B). Differences in morphology between BiH005-A and iXM001 cell clusters became strikingly apparent after reaching the pancreatic progenitor stage. For instance, iXM001 cell clusters reacquired a rounded structure between day 14 and day 19, whereas BiH005-A cell clusters displayed an irregular shape at the endocrine progenitor stage (Fig. 13B). Furthermore, by the end of the maturation stage, at day 19 of the differentiation, several BiH005-A clusters fused together (Fig. 13B).

To determine whether these morphological changes reflect successful differentiation of BiH005-A and iXM001 iPSCs into β-like cells, expression of key marker genes was

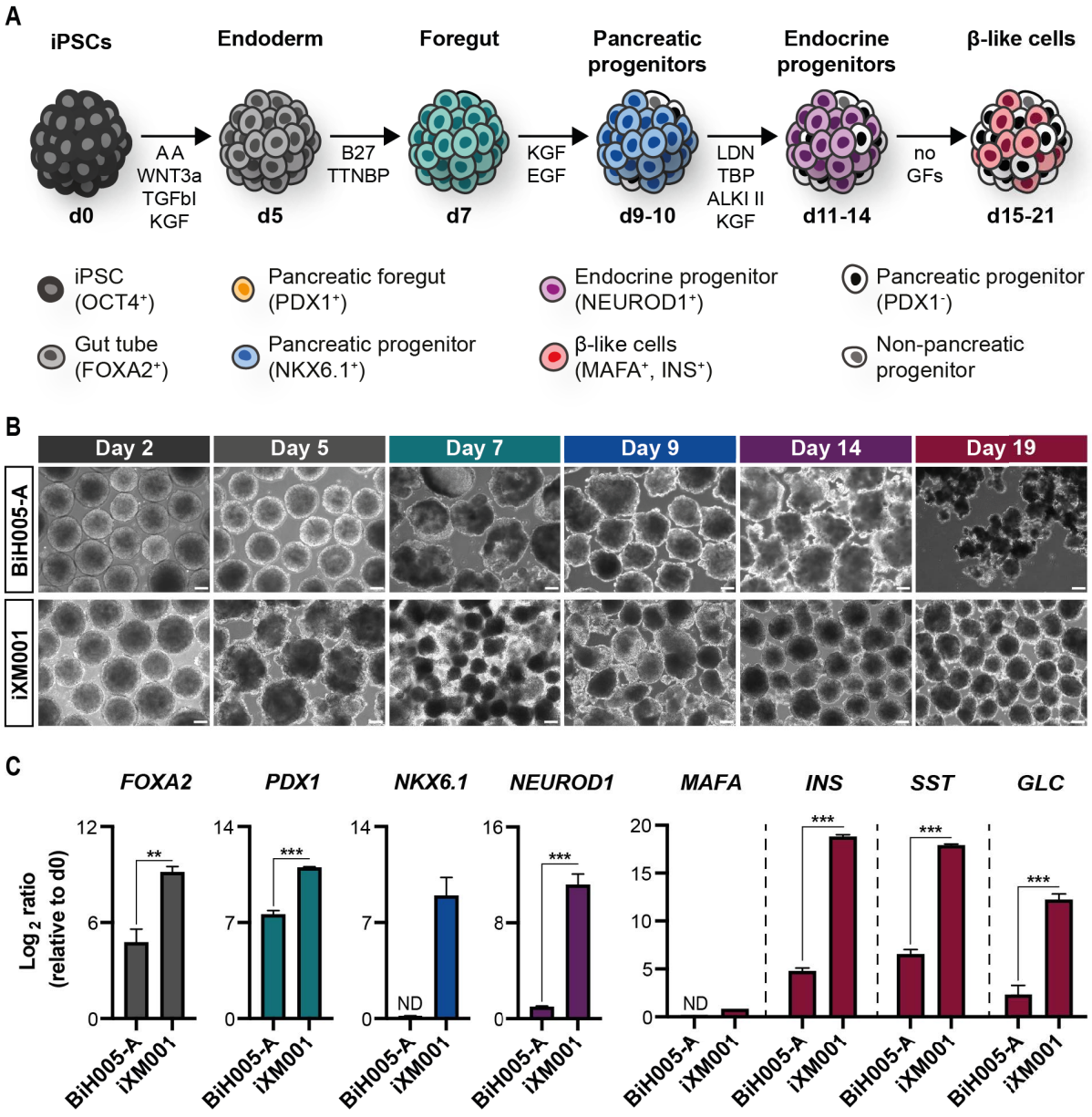

Fig. 13: iXM001 iPSCs represent a good differentiation model. (**A**) Schematic diagram of the procedure used to differentiate human iPSCs into β-like cells. (**B**) Representative bright-field images of iPSC (day 2), endoderm (day 5), foregut (day 7), pancreatic progenitor (day 9), endocrine progenitor (day 14) and β-like cell (day 19)-stages differentiated from BiH005-A or iXM001 cell line, respectively. Scale bar, 100 µm. (**C**) RT-qPCR analyses of indicated stage-specific markers. Values are normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and relative to day 0. Error bars represented as SEM. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.

analysed by RT-qPCR at each stage of the differentiation (Fig. 13C). As expected, the expression of *FOXA2* (endoderm), *PDX1* (pancreatic foregut), *NKX6.1* (pancreatic progenitor) and *NEUROD1* (endocrine progenitor) was significantly induced in iXM001-derived cells at the respective stage (Fig. 13C). Differentiated iXM001 cells at day 19 expressed key pancreatic differentiation markers including *PDX1* and *NKX6.1*, as well as β-cell markers, such as *INSULIN* and *MAFA* (Fig 13C). However, cell clusters also expressed other endocrine

hormones, such as *GLUCAGON* and *SOMATOSTATIN*, indicating the generation of different types of endocrine cells. BiH005-A cells expressed *INSULIN* and other endocrine hormones at the end of the differentiation procedure, but the expression of *MAFA* could not be detected. This could be attributed to the late induction of *NKX6.1* (at day 14, data not shown) as well as to the low induction of *NEUROD1* (day 14), both essential TFs expressed in pancreatic endocrine precursors at day 14 (Fig. 13C). Therefore, the maturation of BiH005-A β-like cells could be delayed.

In summary, I was able to successfully differentiate BiH005-A and iXM001 iPSCs into β-like cells. Moreover, I demonstrated that crucial *in vivo* stages (e.g. pancreatic progenitors and endocrine progenitors) are faithfully recapitulated during differentiation. However, the differentiation of BiH005-A cells is delayed, and BiH005-A-derived β-like cells do not express *MAFA*, which plays a crucial role in the acquisition of glucose-responsive insulin secretion *in vivo*. Thus, iXM001 iPSCs represent a more suitable cell source for setting up a differentiation platform and the protocol described by Russ et al. can be applied without further optimizations.

4.2.2 iXM001 iPSCs are a good model to study differentiation into β-like cells

In the past, several differentiation protocols have been established to generate pancreatic β-cells using distinct cultivation methods, growth factors and small molecules. The most successful approach has been demonstrated to mimic essential steps *in vivo* development *in vitro* through directed stepwise differentiation of human PSCs. However, a common issue during differentiation is the generation of undesired cells which fail to express β-cell markers, such as *NKX6.1* and *PDX1*, co-express multiple endocrine hormones (e.g. *GLUCAGON* and *INSULIN*) or are non-functional after transplantation *in vivo* [18]. Therefore, I characterized the iXM001-derived β-like cells at stage five in further detail with additional assays.

To analyse whether iXM001-derived β-like cells show features of *bone-fide* β-cells, I assessed the expression of key β-cell markers *in vitro* at mRNA and protein level (Fig. 14). As outlined in figure 14A the obtained β-like cells expressed the pancreatic endocrine markers *PDX1* and *NKX6.1* at the end of differentiation, which is a typical feature of pancreatic progenitors and *bona fide* β-cells. Consistently, immunofluorescence analysis revealed that 58.8 ± 2.4% of the cells were double positive for PDX1 and NKX6.1 at day 21 (Fig. 14B, C). Moreover, *NEUROD1* and *NKX2.2*, downstream targets of *NGN3*, were expressed in the differentiated cell clusters from endocrine progenitor stage onwards (Fig. 14A). In addition to *MAFA*, the transcript for the mature human β-cell TF *MAFB* was robustly expressed in the β-like cells. Other genes essential for human β-cell functionality, such as the glucose metabolism enzyme glucokinase (*GCK*), the potassium voltage-gated channel subfamily J member 11

(*KIR6.2*) and the proprotein convertase subtilisin/kexin Type 1 (*PCSK1*) necessary for insulin biosynthesis, were present in the differentiated clusters (Fig. 14A). At mRNA and protein level the β-cell producing hormone INSULIN, as well as other pancreatic hormones, like GLUCAGON and SOMATOSTATIN, could be detected (Fig. 14A). Moreover, roughly 22% of the iPSC-derived β-like cells co-stained for the critical TF NKX6.1 and C-PEPTIDE (Fig. 14D) and thus closely resemble mature human β-cells. Notably, only 3.1% of all differentiated cells co-expressed INSULIN and GLUCAGON, representing polyhormonal cells (Fig. 14E).

To further investigate the functional properties of *in vitro* differentiated β-like cells, I performed a glucose-stimulated insulin secretion (GSIS) assay, in which I measured the release of human insulin at low (about 2 mM) and high (about 20 mM) glucose levels (Fig. 14F). iXM001-derived β-like cells analysed at day 21 responded to an increase in glucose concentration from low to high by secreting 2.2 ± 0.2-fold more insulin. This is comparable with the data reported in the original protocol described in Russ et al. (2015) (1.8 ± 0.9) but lower compared to human islets (8.2 ± 0.2). Therefore, iPSC-derived β-like cells are capable of undergoing GSIS and closely resemble their *in vivo* counterparts.

In conclusion, the majority of the iXM001-derived insulin-producing cells demonstrated co-expression and nuclear localization of TFs critical for β-cell function (*PDX1*, *NKX6.1*, *MAFA*) and only very few were polyhormonal (INS^+/GLC^+). Like previously reported the overall yield of successful differentiated β-like cells ($NKX6.1^+/INS^+$) was about 22% and the β-like cells secreted endogenous insulin in response to changes in physiological glucose concentrations. Therefore, the suspension-based direct differentiation approach can be used to faithfully recapitulate human β-cell development *in vitro* and importantly is applicable to investigate in more detail β-cell development and function in a disease-model context (Fig. 14G).

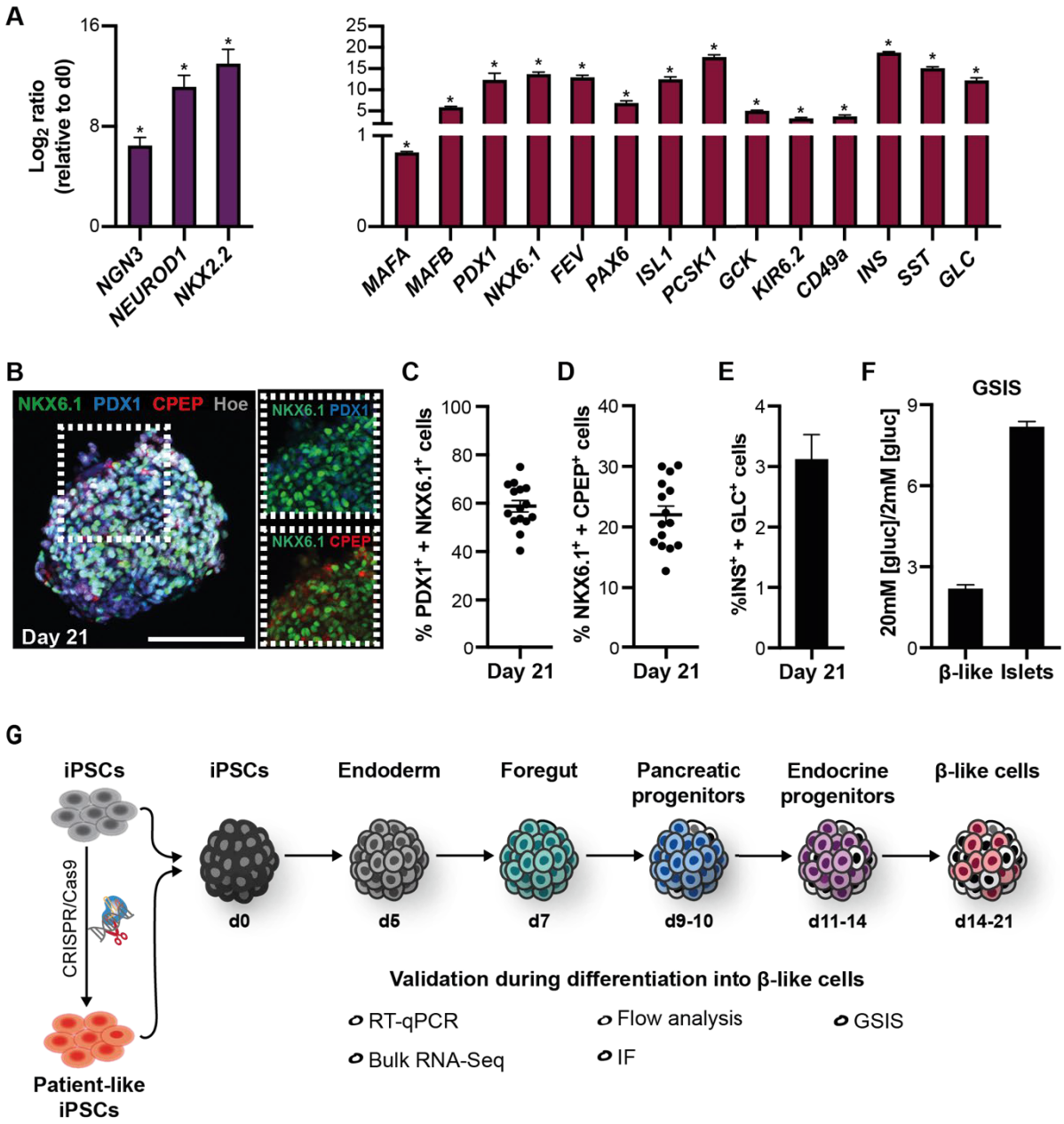

Fig. 14: iXM001-derived β-like cells exhibit key features of mature human β-cells. (**A**) RT-qPCR analyses of endocrine and β-cell specific markers. Values are normalized to GAPDH and relative to day 0. Statistical significance calculated using two-tailed Student's t-test. *p < 0.05. (**B**) Representative whole-mount immunofluorescence staining of differentiated cluster at day 21 for PDX1, NKX6.1 and human C-PEPTIDE (C-PEP). (**C**) Quantification of the percentage of cells positive for PDX1 and NKX6.1, or (**D**) double positive for NKX6.1 and human C-PEPTIDE at day 21, respectively. (**E**) Quantification of the percentage of INSULIN and GLUCAGON double positive cells, representing polyhormonal cells at day 21. Values are average + SEM. (**F**) Representative GSIS of β-like cells at day 21 and human islets. Y-axis indicates ratio of insulin secreted in low glucose conditions to that secreted in high glucose conditions. Values are average + SEM. (**G**) iPSC-based differentiation platform to study intrinsic and extrinsic factors. To investigate the role of the gene candidates in pancreatic cell fate decisions and β-cell function, the Cas9-mediated genome editing in combination with iPSC directed β-cell differentiation is used. Throughout the differentiation phenotypic differences between control, patient-like or treated cells will be assessed at RNA (RT-qPCR, bulk RNA-Seq) and protein (flow analysis, IF) level. Moreover, functionality of iPSC-derived β-like cells will be determined by GSIS.

4.3 Study of the role of HDAC4 during pancreatic β-cell development

4.3.1 Generation of iPSC lines harbouring the *HDAC4* patient-specific variants

The genetic background of the somatic cell donor has been shown to affect the capability of reprogrammed iPSCs to differentiate efficiently into the desired cell type. Therefore, differences in the differentiation potential of iPSCs derived from patients and healthy donors might disguise the mutation-specific phenotype. To overcome this problem and to investigate distinct putative diabetes-linked mutations in parallel without the requirement to generate a large number of isogenic controls with corrected mutations, I introduced the identified patient-specific variants into the same iPSC background. Moreover, this supports the study of multiple mutations without the need for patient recruitment and the differences in differentiation response of the iPSC line. Taken together this experimental strategy maximized the sensitivity for phenotypic changes and allowed me to directly compare different variants.

In order to engineer iPSC lines carrying the identified patient variants, I employed a previously established inducible CRISPR-Cas9 system (iCRISPR) in iXM001 iPSCs. This system includes a DOX-inducible Cas9 and a plasmid carrying the sgRNA together with a Venus fluorescent reporter gene, which enables the enrichment of transfected iPSCs by fluorescence-activated cell sorting (FACS) (Fig. 16A). The point mutation of interest can be introduced through HDR using ssODNs. After FACS, single cells are expanded to form colonies that are screened for recombination with the donor template. Positive clones are validated by Sanger sequencing and overall the whole procedure takes about one month (Fig. 15A).

The described iCRISPR system is very powerful and in combination with directed differentiation allows to dissect the function of gene candidates in the context of β-cell development and function. Therefore, I used the CRISPR-Cas9 genome-editing technology in iXM001 cells to generate patient-like iPSCs harbouring the three gene variants (*HDAC4* c.A800G, *GLI1* c.G119G and *GLI2* c.C4661T), which were selected based on the *in silico* data and preliminary characterization (Table 13).

The gene editing strategy undertaken to target the *HDAC4* locus and insert the patient variants in exon 7 is shown in figure 15B. A designed guide RNA targeting the *HDAC4* locus near the mutation site was used in combination with a ssODN of 120 bases as a donor template to introduce the desired point mutation by HDR (Fig. 15B). I isolated successfully transfected iXM001 iPSCs expressing the sgRNA cloned into a plasmid carrying the Venus reporter by FACS (Fig 15D). After single-colony expansion I genotyped over 200 clones (Fig.

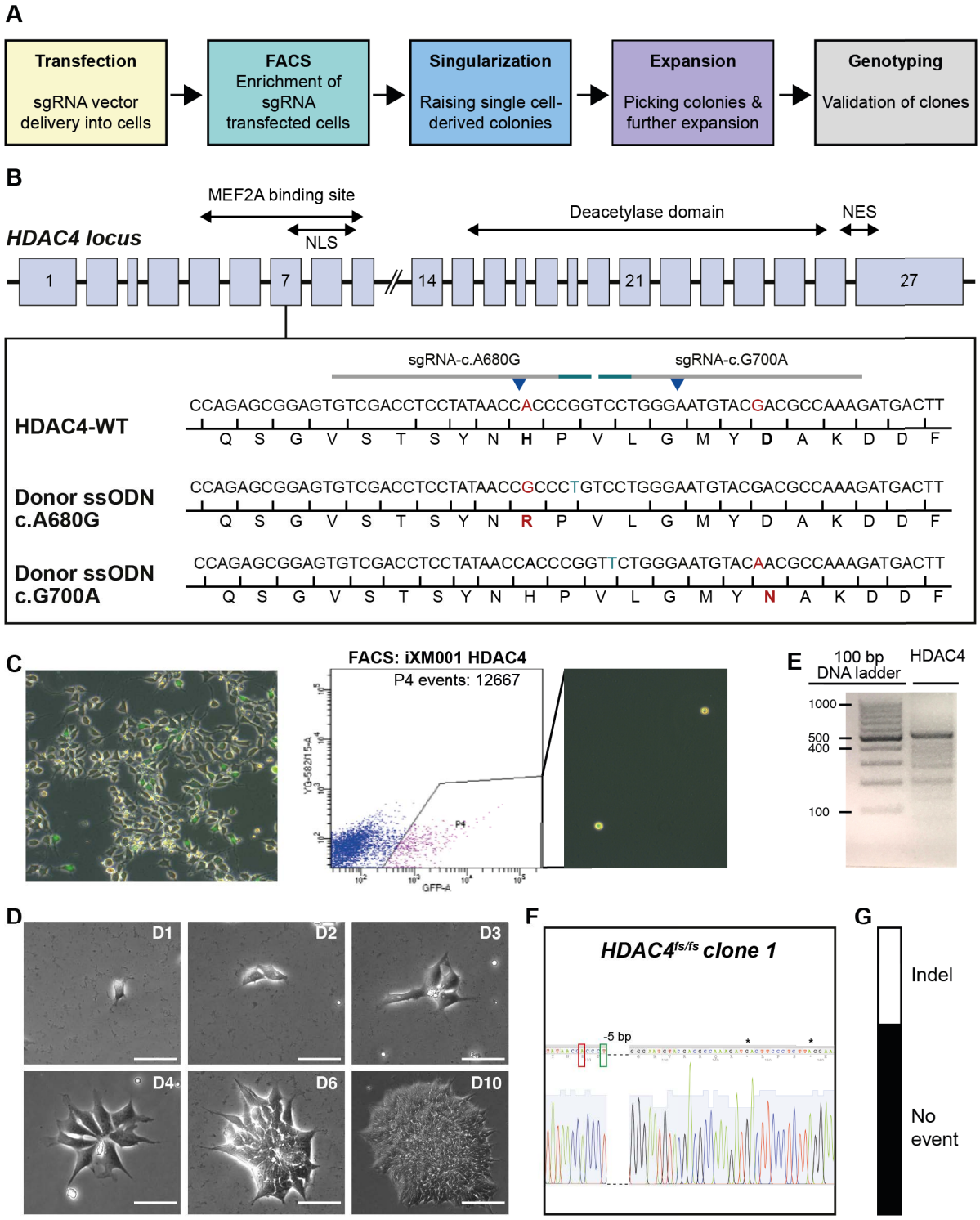

Fig. 15: Generation of patient-like iXM001 iPSC lines. (**A**) Overview of the workflow for CRISPR-Cas9-mediated generation of patient-like iPSC lines. (**B**) CRISPR sgRNA design for generating HDAC4 c.A680G or c.G700A disease variant using the iXM001 line. The target sequence of the sgRNA and the protospacer-adjacent motif (PAM) sequences are indicated in grey and green, respectively. The mutation was introduced through homology direct repair using a ssODN template. The blue arrow indicates the predicted Cas9 cleavage site. (**C**) FACS enrichment of transfected iPSCs. (**D**) Single colony expansion at 1, 2, 3, 4, 5 or 10 days after FACS sorting, respectively. Scale bar, 500 µm. (**E**) Representative PCR amplicon used for sequencing clones. (**F**) Designed CRISPR/Cas9 strategy resulted in the generation of potential *HDAC4* knockout cell lines. DNA sequencing data for clone 1 is illustrated, showing deletion of 5 base pairs (bp). Frameshift mutations are marked by asterisks. (**G**) CRISPR-Cas9 efficiency.

15E and F). Even though, the gene-editing approach was repeated three times with distinct sgRNAs, it did not succeed in the establishment of a patient-like iPSC clone carrying any of the two variants in *HDAC4* (Fig. 15G). Only one screened clone carried the heterozygous mutation *HDAC4 c.A800G,* but also had additional indels rendering it unsuitable for further studies. Nevertheless, over 30% of the screened *HDAC4* clones harboured frameshift mutations caused by an average deletion of five base pairs in close proximity to the double strand break site (Fig. 15G and H). Moreover, in several clones the additional silent mutation to destroy the PAM recognition site was inserted, confirming that homologous repair occurred but it was accompanied by undesired CRISPR-induced deletions. These cell lines can be used for loss-of-function studies to examine the consequences of *HDAC4* deletion during β-cell differentiation. In parallel, the same procedure was successfully applied to engineer patient-like iPSC clones for the selected variants in *GLI1* and *GLI2*, respectively.

In conclusion, the combination of a DOX-inducible Cas9 iPSC line with a sgRNA/reporter-plasmid can successfully be used to generate patient-like mutant cell lines. However, gene-editing efficiency highly relies on occurrence of HDR and the proximity of the sgRNA-mediated cutting site to the mutation.

4.3.2 Generation of an inducible *HDAC4* knockdown iPSC line to dissect the function of *HDAC4* during β-cell development

Epigenetic modulators, like the class IIa histone deacetylase HDAC4, have been highlighted in previous studies to regulate developmental aspects and implied to control pancreatic lineage decisions to some extent. Specifically, the regulatory mechanisms and temporal activity of HDAC4 during β-cell development is unknown. To address this in humans and complete the functional studies in *Xenopus* embryos and Min6 cells, I decided to undertake a CRISPR interference (CRISPRi) approach.

The CRISPRi model system is based on a DOX-inducible deactivated Cas9 fused to a KRAB repression domain, allowing specific and reversible knockdown of gene expression in iPSCs and derivatives (Fig. 16). Thus, it represents an alternative approach for loss-of-function studies that inhibits expression by guiding a transcriptional repressor to the transcription start-site of target genes. To determine if this method can be applied during differentiation into β-like cells and regulates the temporal expression window of *HDAC4* in more detail, I differentiated the CRISPRi iPSCs towards β-like cells as described previously. Like iXM001 iPSCs, CRISPRi iPSC lines formed round clusters, which progressed efficiently through endoderm (day 5), pancreatic foregut (day 7), pancreatic progenitor (day 9) and finally endocrine progenitor (day 14) stage, where they expressed *PDX1*, *NKX6.1* and *NEUROD1* (Fig. 16F). In comparison to the iXM001 iPSC line, the differentiation toward β-like cells was

less efficient and needs further optimization. However, a detailed stage-specific analysis of *HDAC4* expression revealed its defined induction at pancreatic progenitor stage (Fig. 16F). Also, in other iPSC lines (e.g. iXM001, data not shown) the expression of *HDAC4* was induced at pancreatic progenitor stage and remained high in endocrine progenitor cells. Therefore, the CRISPRi system represents an ideal model to investigate the biological function of *HDAC4* during pancreatic progenitor specification or endocrine progenitor formation, respectively. This can be achieved by treating the cells with DOX at distinct time-points during differentiation (Fig. 16E).

In a first attempt to generate a stable *HDAC4* knockdown cell line, two different gRNAs targeting the first exon (HDAC4-gRNA$_{238}$ and HDAC4-gRNA$_{454}$) of *HDAC4* were cloned into the mKate expression vector (Fig. 16A and B). These gRNAs were chosen, since they were the closest possible to the transcription start site (TSS). Indeed, in close proximity of the TSS an appropriate PAM sequence was missing, or the GC content was to high decreasing the efficiency of the guide RNA. As a positive control, a published OCT4-gRNA was used to knockdown the expression of OCT4, a core pluripotency TF (REF). After transfection of the gRNA-expression vector into the CRISPRi iPSC line, the blasticidin antibiotic selection was applied to enrich for cells with stably integrated gRNA-expression constructs. After 10 days stable clones appeared as marked by the expression of mKate in over 90% of the cells (Fig. 16C). The knockdown clones were then pooled, expanded and treated with DOX, which induced the expression of dCas9-KRAB-mCherry as indicated by the expression of mCherry. The knockdown efficiency of *OCT4* and *HDAC4* was analysed by RT-qPCR after treatment for four consecutive days with DOX. Bulk populations containing the OCT4-specific gRNA showed a 2-fold Log knockdown of the *OCT4* target gene by RT-qPCR (Fig. 16D). Consistently, cells started losing their stem cell characteristic morphology and spontaneously differentiated (data not shown). In contrast, knockdown of *HDAC4* by any of the two gRNAs had no effect on the expression of *OCT4* and cells showed normal pluripotent morphology. However, the expression of *HDAC4* was slightly repressed by HDAC4-gRNA$_{238}$. The other HDAC4 gRNA did not alter the expression of *HDAC4* and is thus not suitable to generate a stable knockdown cell line for *HDAC4*.

Taken together, the CRISPRi system is a powerful tool to functionally dissect the biological roles of gene candidates during pancreas development. In addition, it allows to precisely and temporally defined knockdown the transcript of interest. However, the results presented in my thesis underscore the need to test multiple gRNAs to reach a robust knockdown efficiency. The HDAC4-gRNA$_{238}$ appears as a promising gRNA to knockdown the expression of *HDAC4*. Current investigations are ongoing to characterize the putative *HDAC4* knockdown iPSC line in more detail. These will include Western blot analysis to assess the

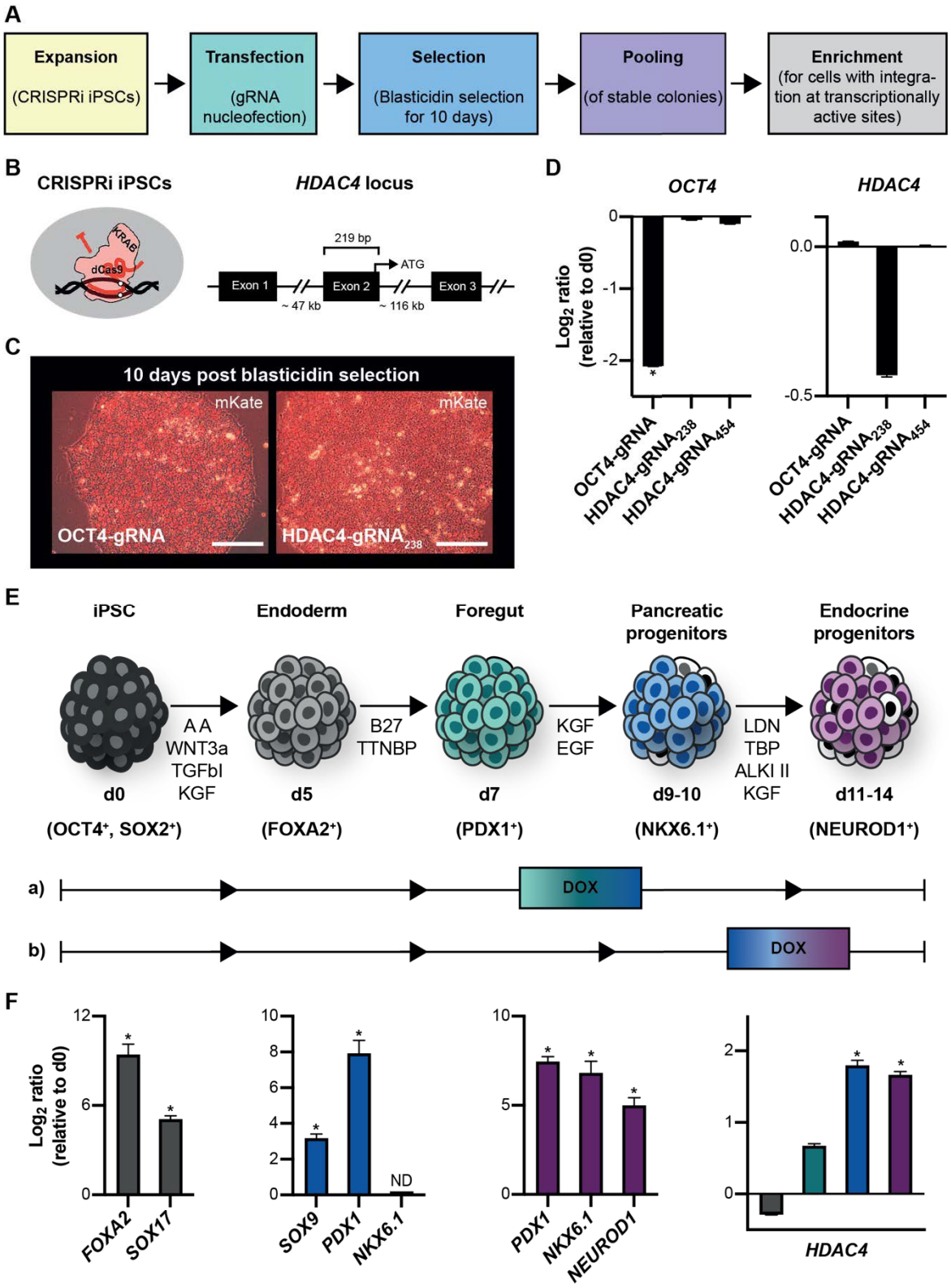

Fig. 16: Establishing an inducible *HDAC4* knockdown iPSC line. (**A**) Schematic overview of workflow to engineer inducible *HDAC4* knockdown iPSC lines. (**B**) Illustration of the CRISPRi iPSC line and genomic locus of *HDAC4* to design gRNA. The CRISPRi iPSC line harbours a DOX-inducible dCas9-KRAB. The dCas9 fusion protein pairs with a gRNA to repress expression from target genes. (**C**) IF of mKate ten days after blasticidin selection in *OCT4* or putative *HDAC4* knockdown iPSCs, respectively. Scale bars, 20 µm. (**D**) RT-qPCR of *OCT4* and *HDAC4* in iPSCs treated for four days with DOX. (**E**) Schematic diagram of the protocol used to differentiate CRISPRi iPSCs into endocrine progenitors. Potential *HDAC4* knockdown time-points are shown (a and b). (**F**) RT-qPCR of stage-specific markers and *HDAC4* during differentiation into endocrine progenitor cells. *p < 0.05.

knockdown of *HDAC4* at the protein level. Furthermore, the expression of *HDAC4* increased during differentiation towards β-like cells, therefore the DOX-treatment requires fine tuning to address the potential functions of *HDAC4* at distinct developmental stages.

4.4 Study of the role of the putative pathogenic variant c.C119G in *GLI1* during β-cell development

The heterozygous variant c.C119G in exon 3 of *GLI1* was confirmed through Sanger sequencing in one patient of our unique cohort. Mutations in any other established MODY gene were not present. The patient presented hyperglycaemia and was diagnosed with diabetes at the age of 13 years: Insulin therapy was started. Moreover, mother and maternal grandmother have T2D. So far mutations in *GLI1* have not been associated with diabetes. The identified amino acid substitution (p.P168R) was predicted to be deleterious by multiple computational algorithms and preliminary functional assays showed impaired transcriptional activation potential of GLI1 mutant variant. To study the role of this putative pathogenic variant in human β-cells, I engineered an iPSC line carrying the respective GLI1 c.C119G mutation and differentiated it *in vitro* to β-like cells.

4.4.1 The variant *GLI1* c.C119G can be introduced by CRISPR-Cas9 in human iPSCs

Employing the previously described CRISPR-Cas9 genome editing approach, I introduced the c.C119G mutation into the iXM001 iPSC line from a healthy individual. For this purpose, I used an sgRNA, which cuts two base pairs away from the site of the mutation and a repair ssODN containing the mutated nucleotide in the WT position (red letter) as well as a synonymous point mutation to destroy the PAM recognition site (green letter) (Fig. 17A). After transfection, FACS of Venus^{+} cells and genotyping of the recovered single cell iPSC colonies, I established an iXM001 clonal line carrying the heterozygous C to G variant in exon 3 of *GLI1*. The clone displayed a typical round shape ESC-like morphology and growth behaviour comparable to the unmodified WT iPSC line. Expression of hallmark pluripotency markers, such as *OCT4*, *SOX2* and *NANOG,* was observed at RNA and protein levels (Fig. 17E and F). In addition, the clone expressed the pluripotent surface marker SSEA4 (data not shown) and was positive for alkaline phosphatase activity (Fig. 17D). The clone that harbours the heterozygous mutation c.C119G in *GLI1* is henceforth referred to as GLI1$^{+/-}$ (Fig. 17).

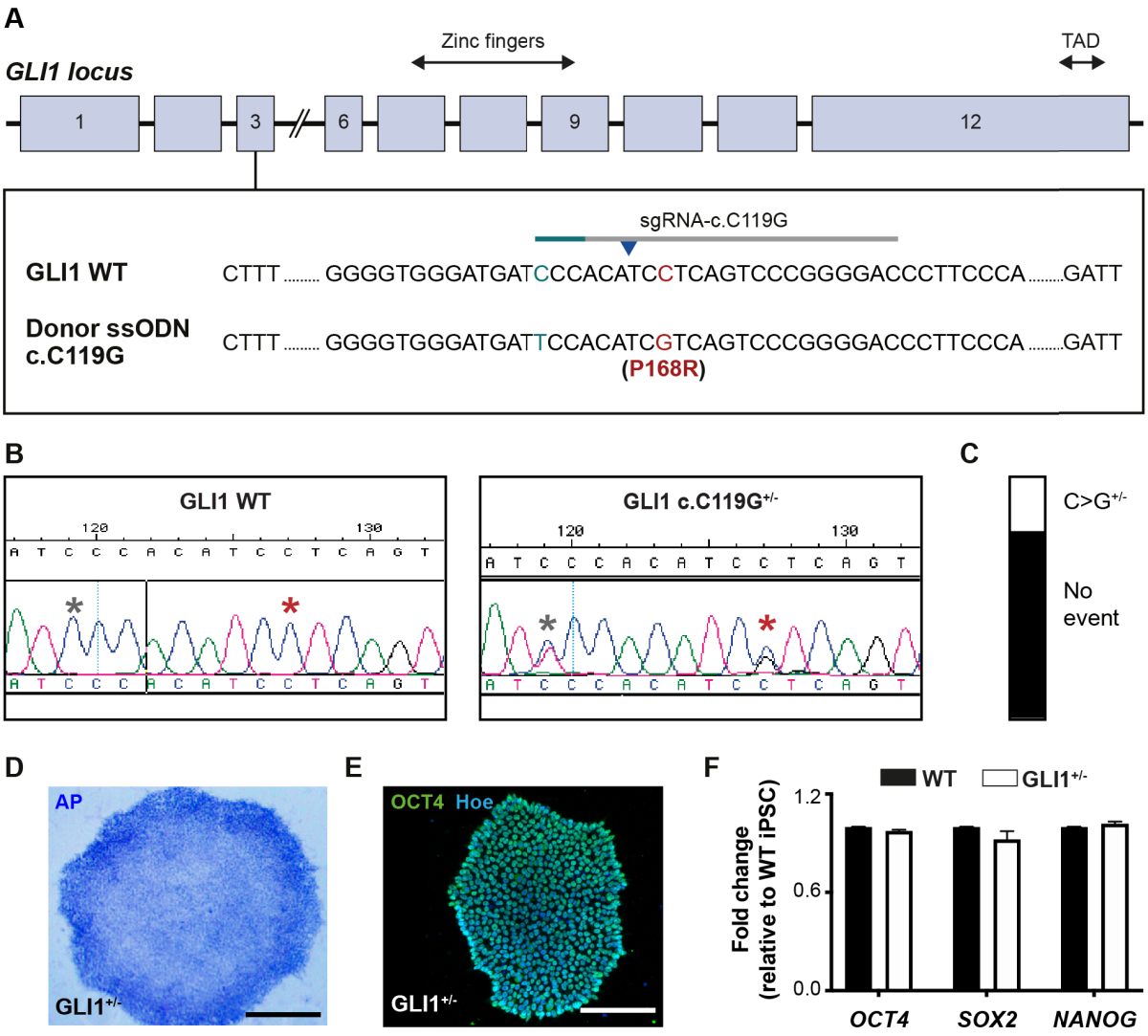

Fig. 17: Generation of a patient-like GLI1 c.C119G iPSC line. (**A**) CRISPR sgRNA design for generating GLI1 c.C119G disease variant using the iXM001 line. The target sequence of the sgRNA and the protospacer-adjacent motif (PAM) sequences are indicated in grey and green, respectively. The mutation was introduced through homology direct repair using a ssODN template. The blue arrow indicates the predicted Cas9 cleavage site (**B**) Sequencing-graphs of GLI1 WT and heterozygous GLI1 c.C119G mutant line. Red asterisk indicates C > G switch. Grey asterisk indicates silent point mutation in the PAM recognition site (**C**) CRISPR Cas9 efficiency. (**D**) Alkaline phosphatase (AP) staining of generated GLI1$^{+/-}$ iPSC line. (**E**) IF of OCT4 in GLI1$^{+/-}$ iPSC line. (**F**) RT-qPCR analysis of selected pluripotency gene transcripts in WT and CRISPR-Cas9 engineered GLI1$^{+/-}$ iPSC line. Values are normalized to GAPDH and relative to WT iPSCs. Error bars represented as SEM. n=3. Scale bars = 25 µm.

4.4.2 Characterization of the impact of *GLI1* c.C119G$^{+/-}$ variant on β-like cell differentiation

To investigate if the *GLI1* variant results in a disease relevant phenotype during β-cell development, I differentiated the patient-like iPSC line into β-like cells. The WT iPSC line was used as reference control. Mutant and control iPSC lines formed clusters comparable in size and numbers after sphere induction (data not shown). Furthermore, both lines differentiated

efficiently into definitive endoderm on day 5, as shown by the expression of *FOXA2* and *SOX17* (Fig. 18A). The induction of *SOX17* was slightly delayed in *GLI1* mutant cell clusters. However, this did not alter the induction of *PDX1* or *NKX6.1* and thus the progression into pancreatic progenitor stage on day 9 (Fig. 18B).

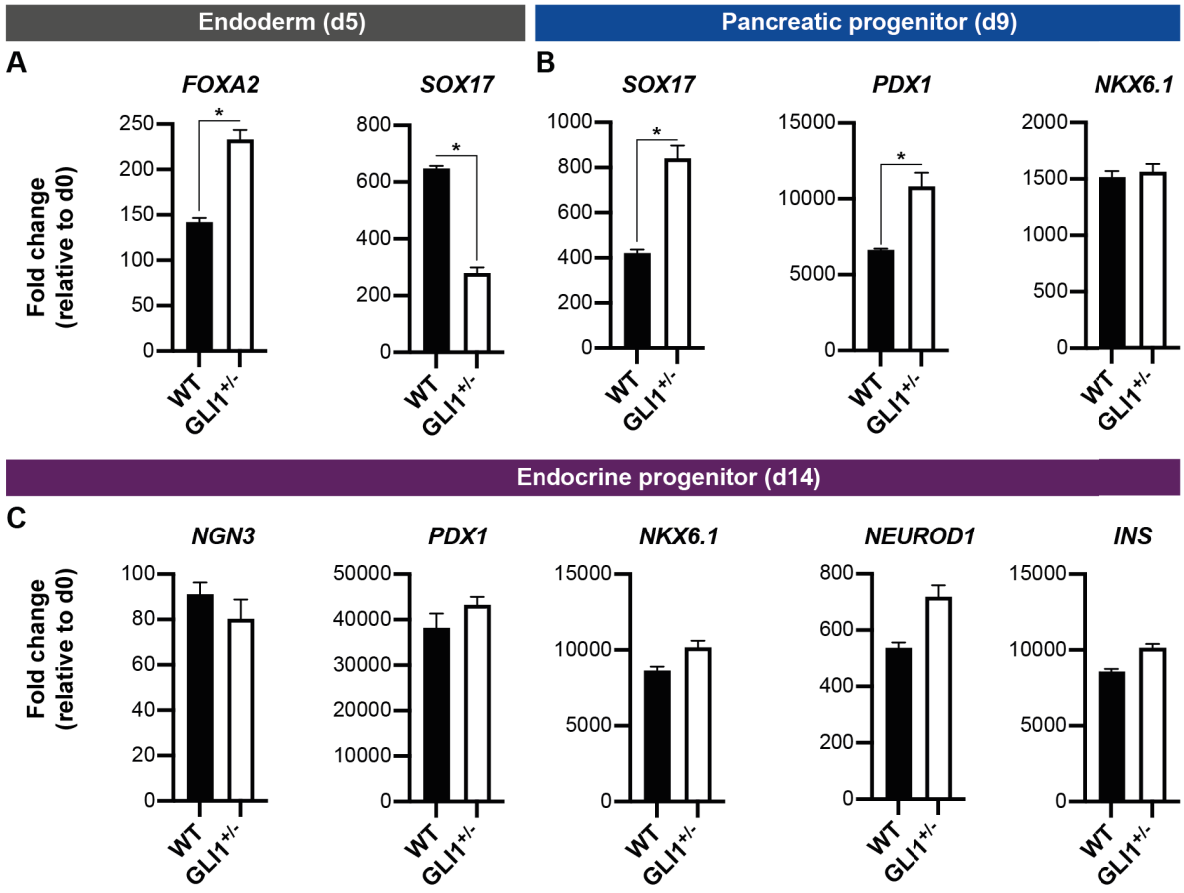

Fig. 18: Characterization of GLI1$^{+/-}$-derived cells during specification of endocrine progenitor cells. RT-qPCR analysis of selected gene transcripts in differentiated cells at D5 (**A**), D9 (**B**) and D14 (**C**). Values are normalized to GAPDH and relative to undifferentiated d0 iPSCs. Error bars represented as SEM. n=1. *p < 0.05; Student's t test.

Following exposure to the endocrine differentiation cocktail for 5 days, *NGN3* and its downstream target *NEUROD1* were induced in mutant- and WT-derived endocrine progenitor cells. Moreover, *PDX1*, *NKX6.1* and *INSULIN* mRNA levels were unaffected by the patient-like mutation in *GLI1* (Fig. 18C). Consistently, the mutant endocrine progenitor cells could further differentiate into β-like cells. Also, at this stage no significant differences in the gene expression profile was determined by RT-qPCR between control and mutant β-like cells. Key genes involved in β-cell differentiation and establishing β-cell identity, such as *PDX1*, *NKX6.1*, *NEUROD1*, *NKX2.2*, *MAFA*, *MAFB* and *FEV*, were all expressed at levels similar to those detected in WT β-like cells. Importantly, genes required for human β-cell functionality,

including *PCSK1*, *KIR6.2* and *SUR1*, were induced in mutant β-like cells. Furthermore, *INSULIN* and other endocrine hormones like *SOMATOSTATIN* and *GLUCAGON* were expressed at the end of the differentiation (Fig. 19).

Altogether, these data indicate that β-like cells could be derived from GLI1 c.C199G$^{+/-}$ iPSCs. Even though, only a single clone was analysed, the results suggest that the putative pathogenic variant in *GLI1* does not diminish the expression of genes important for β-cell development and maturation. Moreover, throughout differentiation no significant loss in the number of mutant cell clusters was observed. Thus, the mutation in *GLI1* unlikely suppresses proliferation or increases apoptosis during β-cell differentiation. However, further studies are necessary to confirm the expression of key β-cell markers at protein level (e.g. by FACS and IF) and to assess if the derived β-like cells are monohormonal and glucose-responsive.

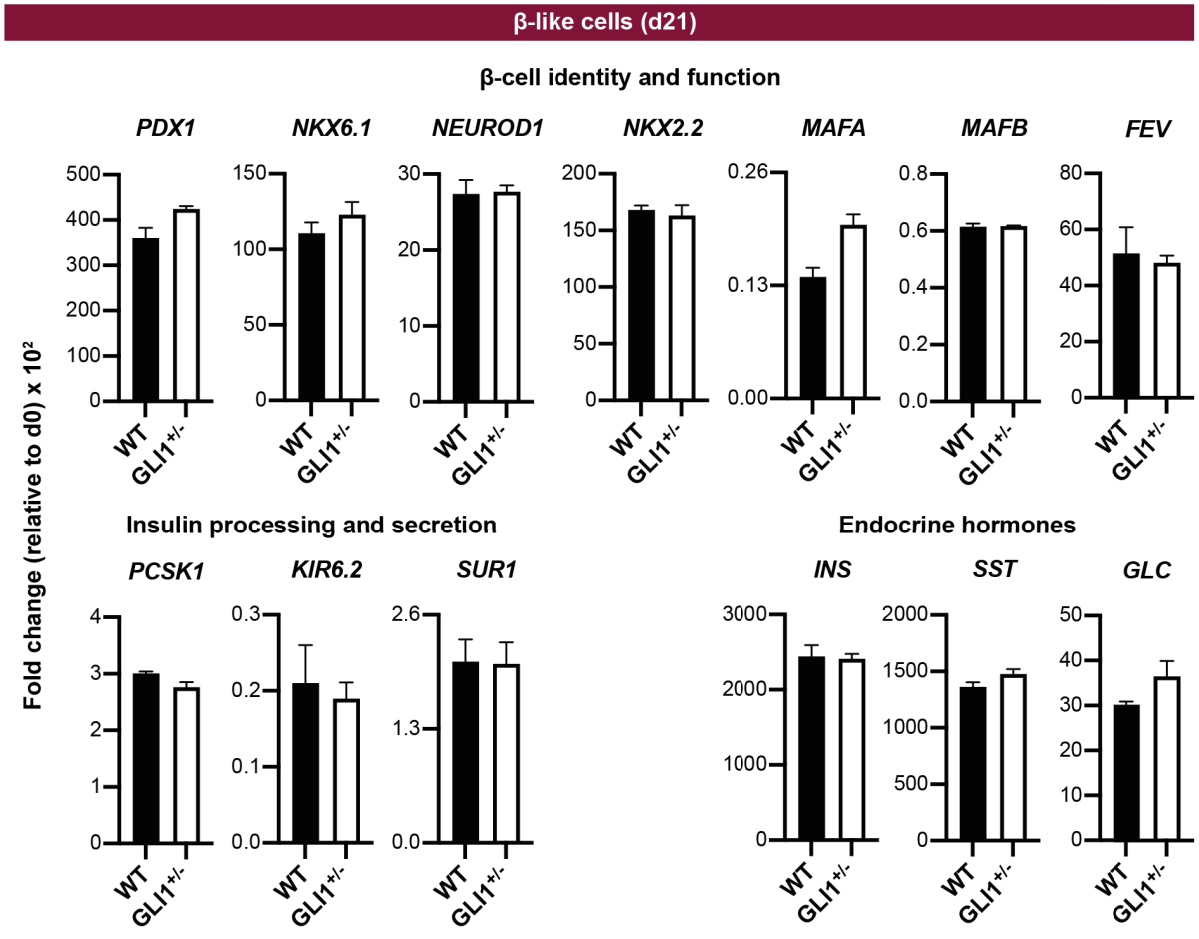

Fig. 19: Gene expression profile of GLI1$^{+/-}$-derived β-like cells closely resembles control cells. RT-qPCR analysis of selected β-cell markers in differentiated cells at D21. Data are normalized to GAPDH and represented as fold change relative to undifferentiated cells (d0). Values shown are mean ± SEM. n=1.

4.5 Study of the role of the putative pathogenic variant c.C4661T in *GLI2* during β-cell development

Mutations in the human *GLI2* gene have been previously reported in association with developmental defects, including holoprosencephaly, a neuroanatomic anomaly resulting from incomplete cleavage of the developing forebrain. Moreover, the zinc-finger TF GLI2 is a downstream target of the SHH signalling pathway, which plays a role during pancreas organogenesis, β-cell formation and function. In several β-cell differentiation protocols the SHH inhibitor cyclopamine is added to the molecular cocktail to induce pancreatic progenitors. Nevertheless, mutations in *GLI2* have so far neither been linked to diabetes nor pancreas development in humans. Here, to study the role of GLI2 in the context of β-cell development, I generated an iPSC line harbouring the novel identified putative pathogenic variant (p.P1554L or c.C4661T) found in diabetic patients using CRISPR-Cas9 genome editing. This GLI2 variant was predicted to be deleterious by five *in silico* prediction programs and impaired the transcriptional activation potential of GLI2 in preliminary *in vitro* assays (Fig. 10).

4.5.1 Identification of the heterozygous variant c.C4661T in *GLI2* in a family with history of diabetes

The heterozygous variant c.C4661T in exon 14 of *GLI2* was identified in all four individuals of a consanguineous family (EgC005) (Fig. 20A). One of the two index children developed diabetes at the age of 5 years and requires up to date multiple daily insulin injections. Currently, the individual is 25 years old and in January 2019 she had an attack of fever and diabetic ketoacidosis. A central line was inserted leading to thrombosis in cardiac vessels. The patient has further diabetic retinopathy in one eye and receives treatment for hypertension and dyslipedimia (e.g. Tritace). The younger sibling, now 23 years old, also carries the variant and has diabetes. He is on insulin therapy but unlike his sister does not have further complications. Although both parents harbour the c.C4661T variant in *GLI2*, only the father has developed diabetes so far. His diabetes improved over the years, the dose of OHAs administrated gradually tapered and for the last two years he has been off therapy. Moreover, the grandmother paternal side had diabetes, but passed away and was therefore not available for sequencing.

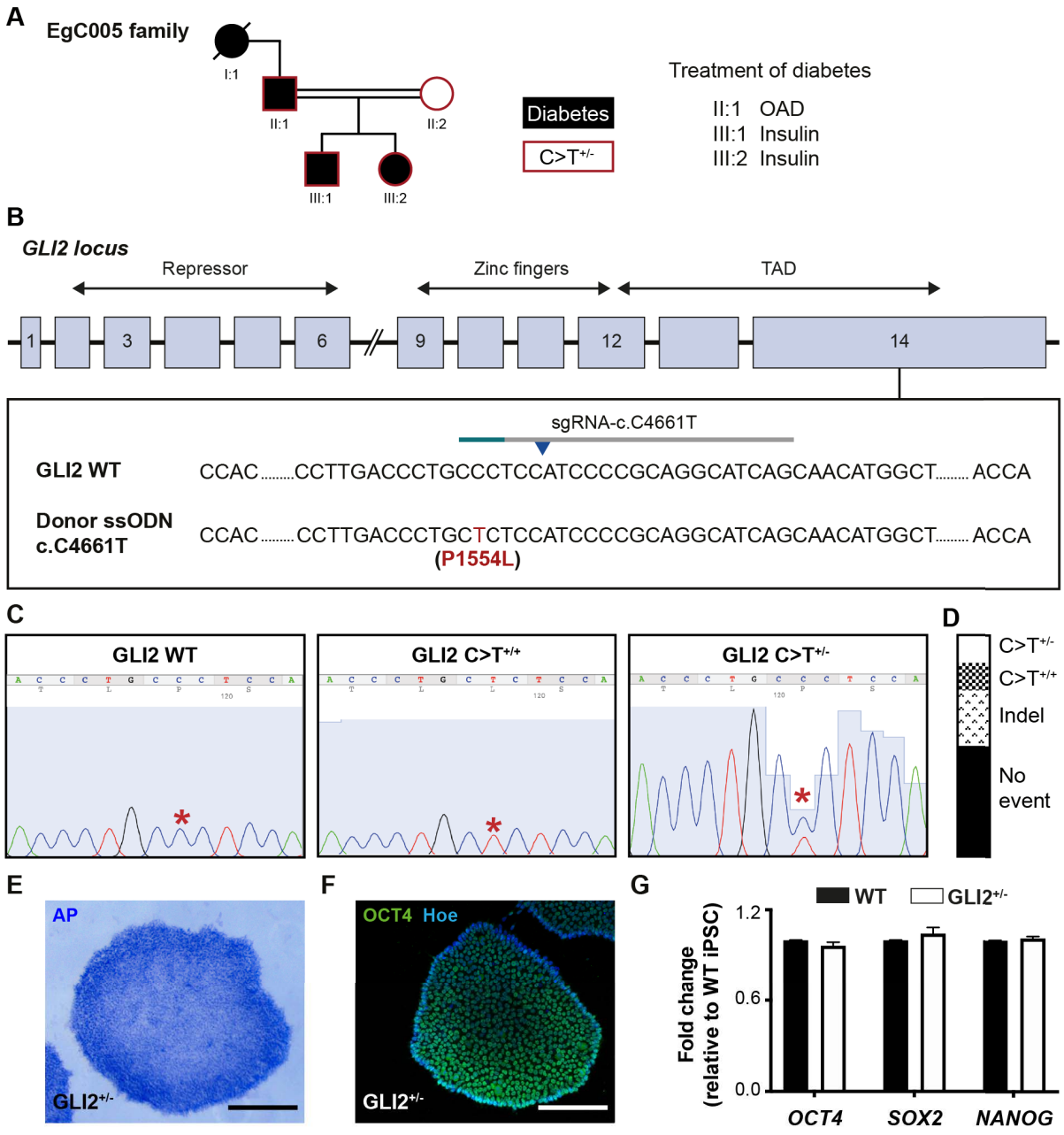

Fig. 20: Generation of patient-like GLI2 c.C4661T iPSC lines. (**A**) Family tree of patients with puberty-onset diabetes and heterozygous variant (c.C4661T) in *GLI2*. Black symbols illustrate diabetic individuals and red symbols illustrate individuals carrying the putative pathogenic variant in *GLI2*. Squares illustrate male subjects and circles illustrate female subjects; a diagonal line through a symbol represents a deceased individual**.** (**B**) CRISPR sgRNA design for generating GLI2 c.C4661T disease variant using the iXM001 line. The target sequence of the sgRNA and the protospacer-adjacent motif (PAM) sequences are indicated in grey and green, respectively. The mutation was introduced through homology direct repair using a ssODN template. The blue arrow indicates the predicted Cas9 cleavage site (**C**) Sequencing-graphs of GLI2 WT, heterozygous and homozygous GLI2 c.C4661T mutant clone. Red asterisk indicates C>T switch. (**D**) CRISPR Cas9 efficiency. (**E**) Alkaline phosphatase (AP) staining of representative GLI2$^{+/-}$ iPSC clone. (**F**) IF of OCT4 in GLI2$^{+/-}$ iPSC clone. (**G**) RT-qPCR analysis of selected pluripotency gene transcripts in WT and CRISPR-Cas9 engineered GLI2$^{+/-}$ iPSC line. Values are normalized to GAPDH and relative to WT iPSCs. Error bars represented as SEM. n=3. Scale bars, 25 µm.

4.5.2 Efficient generation of iPSC lines carrying the patient variant c.C4661T in *GLI2*

The patient variant c.C4661T in *GLI2* was introduced into the WT iXM001 iPSC line with CRISPR-Cas9 and a ssODN carrying the mutation of interest. The inserted mutation directly disrupted the PAM sequence (Fig. 20B). Heterozygous and homozygous c.C4661T mutant iPSC lines were generated, as confirmed by Sanger sequencing, which showed the C to T nucleotide exchange resulting in a P1554L substitution in the TAD domain of *GLI2* (Fig. 20B). To control for potential CRISPR off-targets and line-to-line variations, I analysed two heterozygous and two homozygous mutant iXM001 iPSC lines carrying the c.C4661T point mutation in *GLI2* and compared them to the isogenic control iXM001 iPSC line. All generated iPSC lines expressed *OCT4*, *SOX2* and *NANOG* at high levels and exhibited typical markers of pluripotency, such as expression of SSEA4 and alkaline phosphatase. The mutant line carrying the heterozygous variant c.C4661T$^{+/-}$ in *GLI2* is henceforth referred to as GLI2$^{+/-}$ and the homozygous mutant as GLI2$^{+/+}$. Representative results are shown for one heterozygous GLI2$^{+/-}$ iXM001 clone (clone number 5) (Fig. 20E-G).

4.5.3 The patient-like c.C4661T$^{+/-}$ mutation in *GLI2* impairs differentiation of iPSCs into endocrine progenitor cells

To test the causality and investigate a potential disease-relevant phenotype of the c.C4661T variant in *GLI2* during β-cell development, I differentiated the patient-like GLI2$^{+/-}$ iPSC line into β-like cells (Fig. 21). Mutant and control iPSCs successfully formed clusters comparable in size and number. Furthermore, no striking difference in endoderm differentiation was observed. Apart from *FOXA2*, which was slightly reduced in mutant cells, no difference in the expression of definitive endoderm (*SOX17*) and pancreatic endoderm markers (*SOX9*, *PDX1*) was detected between GLI2$^{+/-}$ mutant- and WT-derived cells (Fig. 21A and B). Heterozygous mutant iPSC lines efficiently differentiated into pancreatic endoderm progenitors and the induction of *PDX1* was not affected. Moreover, above 90% of the cells were PDX1 positive in all iPSC differentiation experiments (n = 3), as assessed by FACS analysis at day 5. For instance, about 93.9% WT- and 97.51% GLI2$^{+/-}$ mutant-derived cells were positive for *PDX1* (Fig. 21A). Additionally, the cells co-expressed GATA6, which was shown to be required in the formation of both definitive endoderm and pancreatic progenitor cells [187,207,365]. Interestingly, I observed in WT- and mutant-derived PDX1^{+} cells a divergence in the expression level of GATA6 (Fig. 21A).

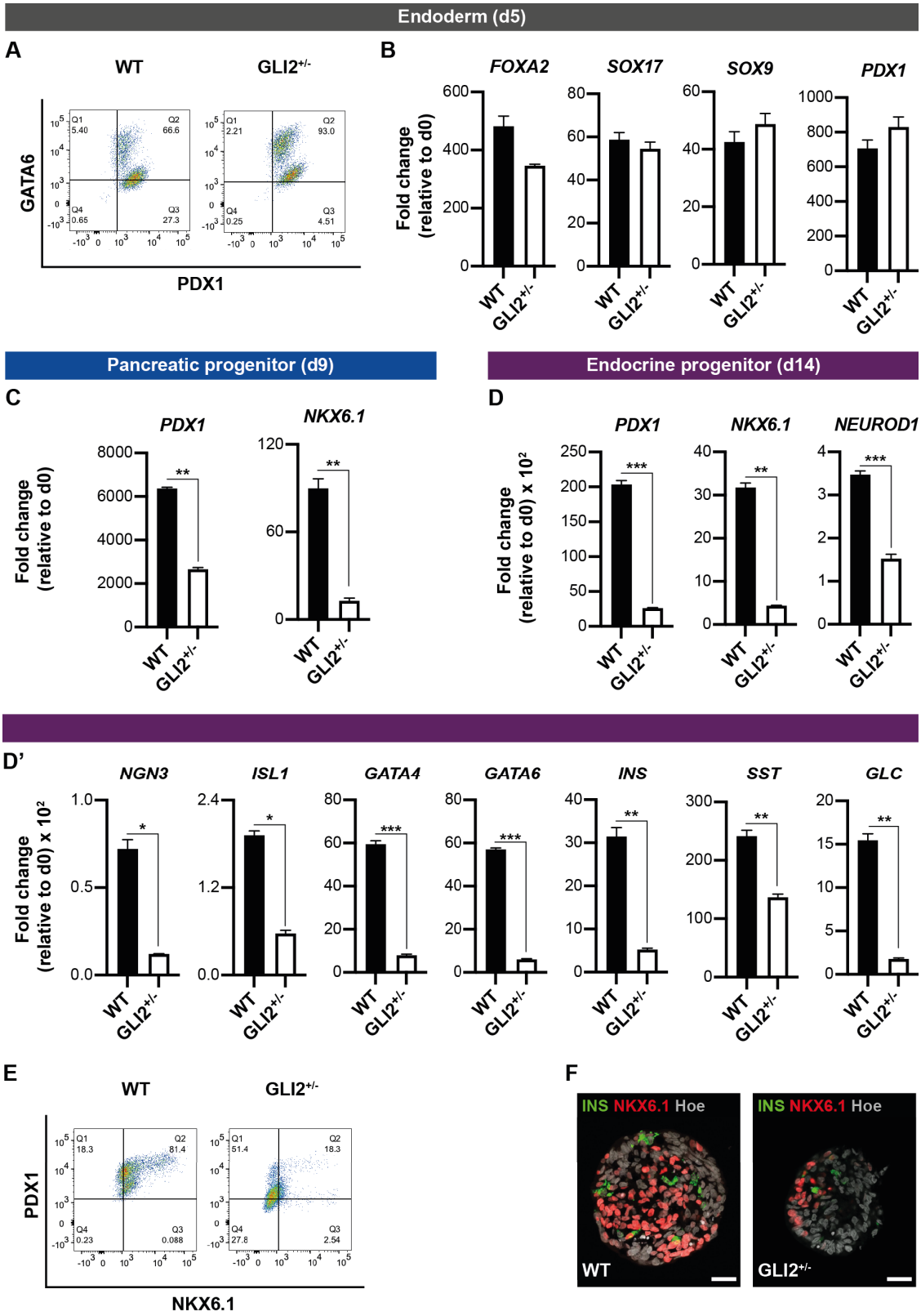

Fig. 21: Characterization of GLI2$^{+/-}$-derived cells during specification of endocrine progenitor cells. (**A**) Representative FACS plot of PDX1^{+} and GATA6^{+} cells in WT- and GLI2$^{+/-}$-derived cells at day 5 during differentiation. RT-qPCR analysis of selected gene transcripts in differentiated cells at D5 (**B**), D9 (**C**) and D14 (**D**). Data are represented as fold change relative to undifferentiated cells (d0). Values shown are mean ± SEM. n=3. *p < 0.05; **p < 0.01; Student's t test. (**E**) Representative FACS plot of NKX6.1^{+} and PDX1^{+} cells in WT- and GLI2$^{+/-}$-derived cells at day 14 during differentiation. (**F**) Immunostaining for NKX6.1 and INSULIN of WT- and GLI2$^{+/-}$-derived endocrine progenitor cells. Scale bars, 20 µm.

However, the ratio between low and high GATA6-expressing cells was highly variable across various differentiation experiments and this observation needs further investigations. Altogether, these data indicate that the putative pathogenic variant c.C4661T$^{+/-}$ in *GLI2* does not impair early pancreatic endoderm differentiation (Fig. 21).

To address whether the point mutation in *GLI2* affects later stages of β-cell development, the patient-like cell clusters were further differentiated towards the pancreatic lineage. Patient-like GLI2$^{+/-}$ iPSCs progressed to the pancreatic (day 9) and endocrine progenitor stage (day 14) like the WT control cells. Independently of the genotype, I detected PDX1 and NKX6.1 double-positive cells at endocrine progenitor stage (Fig. 21E). However, the number of double-positive cells was significantly lower in GLI2$^{+/-}$-derived endocrine progenitors, as compared to control cells, clearly demonstrating that the expression of NKX6.1 and pancreatic differentiation into endocrine progenitor cells was impaired (Fig. 216D-F). Notably, FACS quantification revealed that GLI2$^{+/-}$ mutant lines formed only about 20% of endocrine progenitor cells co-expressing NKX6.1 and PDX1. Compared to the control, this is roughly a quarter of the PDX1^{+}/NKX6.1^{+} population (Fig. 21E). Also, the expression levels of other endocrine markers, like *NGN3* and *NEUROD1*, were reduced in GLI2$^{+/-}$-derived endocrine progenitors (Fig. 21D). Consistently, the transcript level of *GATA4*, *GATA6* and *ISL1* were lower in cells derived from GLI2$^{+/-}$ iPSCs. As a result, the expression of endocrine hormones, including *INSULIN*, *SOMATOSTATIN* and *GLUCAGON* was also diminished in mutant cells. In line with the transcript analysis, immunostaining showed that the number of NKX6.1- and INSULIN-double positive endocrine cells was significantly reduced in GLI2$^{+/-}$-derived cell clusters. Together, these data highlight that the patient variant in *GLI2* impairs the activation of genes essential for endocrine progenitor development.

4.5.4 Expression of key β-cell markers is reduced in GLI2 c.C4661T$^{+/-}$-derived β-like cells

Recent studies have demonstrated that the differentiation of PDX1^{+}/NKX6.1^{-} progenitor cells into PDX1^{+}/NKX6.1^{+} endocrine progenitor cells is a key intermediate step necessary for the generation of glucose-responsive β-like cells. As illustrated above, the patient variant c.C4661T in *GLI2* impaired the differentiation of iPSCs into endocrine progenitors. To investigate the functional consequences of the heterozygous *GLI2* point mutation in β-cell development, the endocrine progenitor cells were cultivated for an additional week to promote the formation of mature β-like cells. At the final stage of differentiation (day 21) significant differences were observed in the expression level of key β-cell markers between GLI2$^{+/-}$- and WT-derived β-like cells. Consistent with findings at earlier stages, GLI2$^{+/-}$ cells showed reduced expression of essential β-cell markers, including genes encoding key TFs (e.g. PDX1, NKX6.1, NEUROD1, NKX2.2, MAFB and FEV), proteins important for

glucose metabolism (e.g. PCSK1, KIR6.2 and GCK), and endocrine hormones (e.g. INSULIN) (Fig. 22A). I further performed a functional assay on iPSC-derived β-like cells. Both WT and mutant β-like cells responded to GSIS. Also, the ratio of insulin secreted in high glucose (16.7 mM) to low glucose (2.8 mM) was higher in mutant β-like cells (WT cells: 2.2-fold; GLI2$^{+/-}$ cells: 3.5-fold). However, the level of insulin secreted from GLI2$^{+/-}$ cells was significantly reduced compared to control cells (Fig. 22B).

In conclusion, these findings support impaired β-cell differentiation and β-cell function in GLI2$^{+/-}$ cells, which might be responsible for a predisposition to diabetes in individuals carrying the c.C4661T variant in *GLI2*.

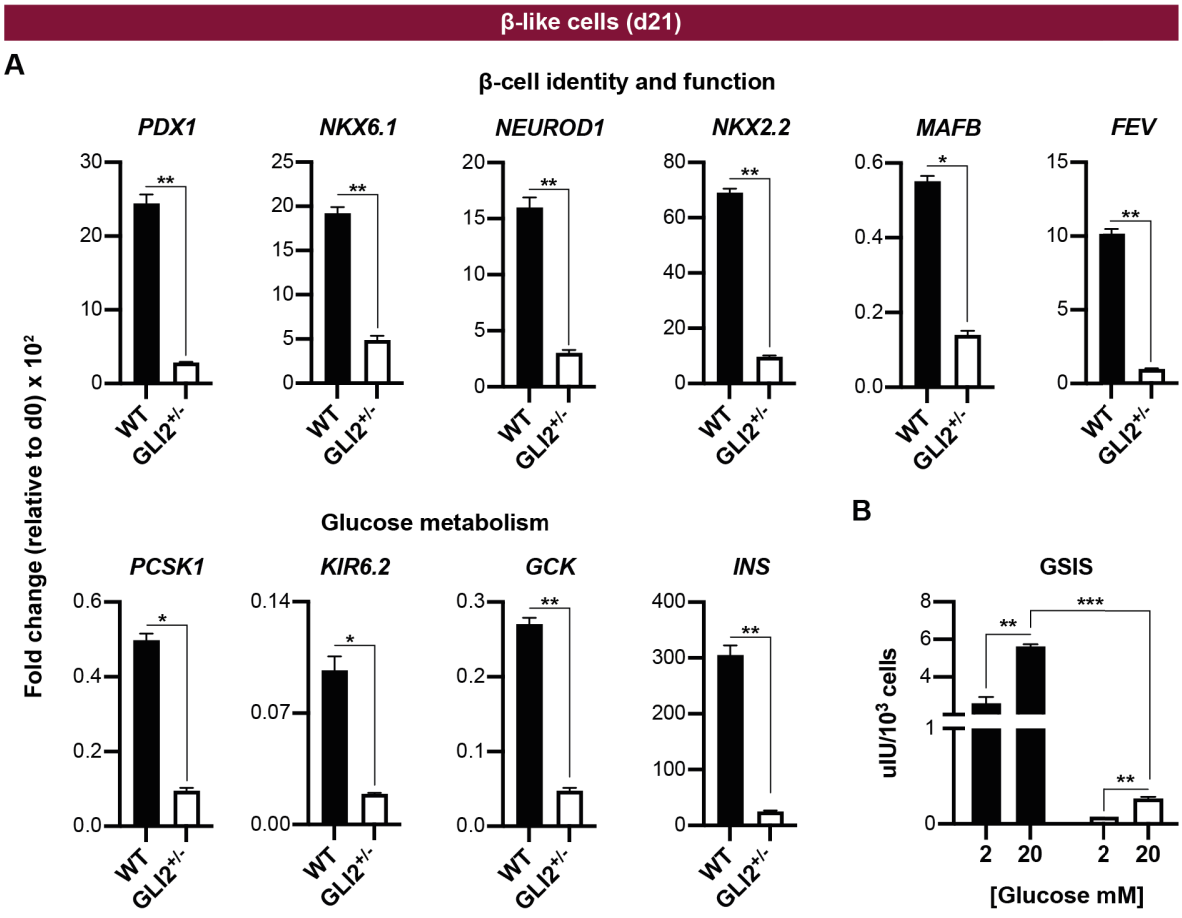

Fig. 22: Characterization of GLI2$^{+/-}$-derived β-like cells. (**A**) RT-qPCR analysis of selected gene transcripts in differentiated cells at D21. Data are represented as fold change relative to undifferentiated cells (d0). Values shown are mean ± SEM. n=3. *p < 0.05. (**B**) GSIS assay of control GLI2$^{+/-}$ derived β-like cells at day 21. Cells were challenged sequentially with low (2 mM) and high (20 mM) glucose with a 60-min incubation for each concentration. One representative experiment is shown. *p < 0.05, **p < 0.01, ***p < 0.001; Student's t test.

4.5.5 Phenotype of *GLI2* c.C4661T is dose-dependent

Gli1-deficient mice do not show apparent phenotypes, are viable and fertile, whereas Gli2 knockout mice die at birth [417,418]. Moreover, several of the reported pathogenic mutations in the human GLI2 lead to a truncated protein with loss of the C-terminal activator domain. Here we identified a novel putative pathogenic variant in GLI2, which impaired pancreas differentiation. To further characterize the phenotype and assess a dose-dependent effect of c.C4661T, the generated homozygous GLI2$^{+/+}$ iXM001 iPSC lines were differentiation towards β-like cells. Mutant and control lines differentiated efficiently into definitive endoderm on day 5 as shown by cell morphology and the expression of *FOXA2* and *SOX17* (Fig. 23B). Similar to GLI2$^{+/-}$-derived cells, the expression of *FOXA2* was slightly reduced in GLI2$^{+/+}$-derived endoderm cells but this did not alter the induction of the pancreatic lineage program, characterized by the expression of *PDX1* and *SOX9*. Quantification by flow cytometry revealed that over 90% of WT and GLI2$^{+/+}$-derived cells were positive for PDX1 (Fig. 23A). Thus, all iPSC lines differentiated normally into endoderm cells committed to the pancreatic lineage since the induction of *PDX1* is neither affected in a *GLI2* c.C4661T heterozygous nor homozygous context.

WT and GLI2$^{+/+}$ cells progressed through foregut (day 7), pancreatic progenitor (day 9) and endocrine progenitor (day 14) stage. However, after endoderm stage, the number of cell clusters gradually decreased from stage to stage and the differentiation of GLI2$^{+/+}$ mutant lines was arrested at endocrine progenitor stage, failing to reach β-like cell stage, which is different from GLI2$^{+/-}$ iXM001 iPSCs. At mRNA level, differences became evident at pancreatic progenitor stage and exacerbated at endocrine progenitor stage. For instance, the expression of *PDX1* and *NKX6.1* diminished significantly in GLI2$^{+/+}$-derived endocrine progenitor cells (Fig. 23C), with only a small number of GLI2$^{+/+}$-derived cells (about 10%) being positive for both PDX1 and NKX6.1 at day 14 (Fig. 23D). Moreover, PDX1 staining intensity was decreased at this point and in some clusters NKX6.1 could not be detected by whole-mount immunostaining (Fig. 23E). Previous studies showed that high co-expression of PDX1 and NKX6.1 in progenitor cells is crucial for an efficient induction of the endocrine progenitor marker *NGN3*, which will specifically generate functional insulin-producing β-like cells. Consistently, the expression level of *NGN3*, its downstream target *NEUROD1*, as well as *GATA4* and *GATA6* were strikingly reduced in GLI2$^{+/+}$ mutant cells. Contrary to GLI2$^{+/-}$-derived endocrine progenitor cells, the expression of *ISL1* was upregulated in GLI2$^{+/+}$-derived cells (Fig. 23C). Finally, *INSULIN* expression was absent in GLI2$^{+/+}$-derived endocrine progenitor cells.

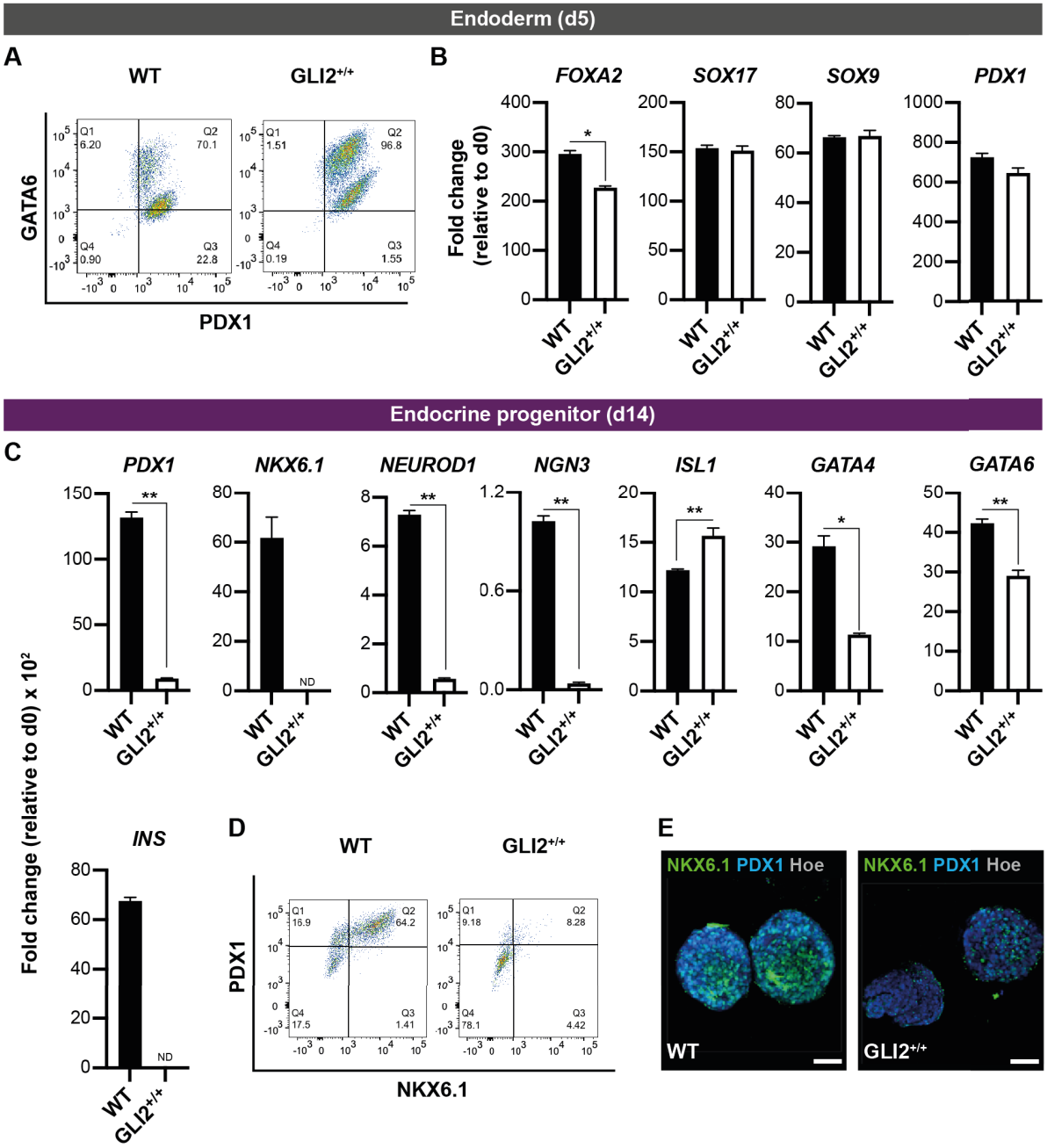

Fig. 23: Characterization of GLI2$^{+/+}$-derived cells during specification of endocrine progenitor cells. (**A**) Representative FACS plot of PDX1^{+} and GATA6^{+} cells in WT- and GLI2$^{+/+}$-derived cells at day 5 during differentiation. RT-qPCR analysis of selected gene transcripts in differentiated cells at D5 (**B**) and D14 (**C**). Data are represented as fold change relative to undifferentiated cells (d0). Values shown are mean ± SEM. n=2. *p < 0.05, **p < 0.01; Student's t test. (**D**) Representative FACS plot of NKX6.1^{+} and PDX1^{+} cells in WT- and GLI2$^{+/+}$-derived cells at day 14 during differentiation. (**E**) Whole-mount immunostaining for the endocrine TFs PDX1 and NKX6.1 of WT- and GLI2 GLI2$^{+/+}$-derived endocrine progenitor cells. Scale bars = 50 µm.

Taking together, the homozygous point mutation in *GLI2* impairs the expression of *NKX6.1* and *NGN3*, which in turn affects endocrine progenitor differentiation. Thus, GLI2$^{+/+}$ iXM001 iPSCs show a more severe phenotype than GLI2$^{+/-}$ iXM001 iPSCs, failing to differentiate into β-like cells and absence of *INSULIN* expression. These findings suggest a

dose-dependent effect of the c.C4661T variant on the endocrine lineage decision. Notably, no patients homozygous for the variant c.C4661T in *GLI2* have been reported so far.

4.5.6 Modulation of the SHH signalling pathway during differentiation of GLI2$^{+/+}$ iXM001 iPSCs

Alternative pancreatic differentiation protocols have been established, in which the SHH signalling pathway is inhibited to enhance the generation of PDX1- and NKX6.1-double positive endocrine progenitor cells. To investigate if modulation of the SHH pathway alters the effects of the variant c.C4661T on β-cell development, I differentiated the GLI2$^{+/+}$ iXM001 iPSC line into β-like cells using a differentiation protocol based on Rezania et al. that includes SHH inhibition [204]. For consistency with the previously used protocol from Russ et al., the differentiation was also carried out in suspension on an orbital shaker [205]. In contrast to Russ et al., the alternative protocol includes two additional intermediate stages of differentiation, namely the primitive gut tube and immature β-like cell stage. Thus, the seven-stage protocol takes one week longer and involves a high number of cytokines and small molecules compared to Russ et al. [204,205]. Specifically, the SHH inhibitor SANT-1 is added to the differentiation medium for eight consecutive days from posterior foregut stage onwards until reaching the endocrine progenitor stage (Fig. 24). This coincides with the time window when significant differences between GLI2 mutant and WT iPSC-derived cells were observed in previous differentiation experiments.

Consistent with previous results, the GLI2$^{+/+}$ and control iPSC lines differentiated efficiently into endoderm (day 2) and progressed to the primitive gut tube stage on day 5 (data not shown). Cell clusters were further differentiated to the endocrine lineage in suspension medium containing, among others, the SHH inhibitor SANT-1. At morphological level, pancreatic (day 10) and endocrine progenitor cell clusters (day 13) derived from GLI2$^{+/+}$ iPSCs were indistinguishable from control clusters at the respective stage. However, the expression of the pancreatic TFs PDX1 and NKX6.1 was lower in mutant pancreatic progenitor cells (Fig. 24B). Similar to the Russ et al. differentiation protocol, the reduced expression of *PDX1* and *NKX6.1* remained until endocrine progenitor stage and was further accompanied by impaired expression of the endocrine markers *NEUROD1* and its upstream activator *NGN3* (Fig. 24C). However, employing the alternative differentiation protocol, GLI2$^{+/+}$-derived endocrine progenitor cells could progress to β-like cells, displaying levels of *PDX1*, *MAFA* and *MAFB* comparable to WT-derived β-like cells. By contrast, the level of expression of *NKX6.1*, *PCSK1*, *KIR6.2* and *INSULIN* was less in GLI2$^{+/+}$-derived β-like cells, highlighting the impaired

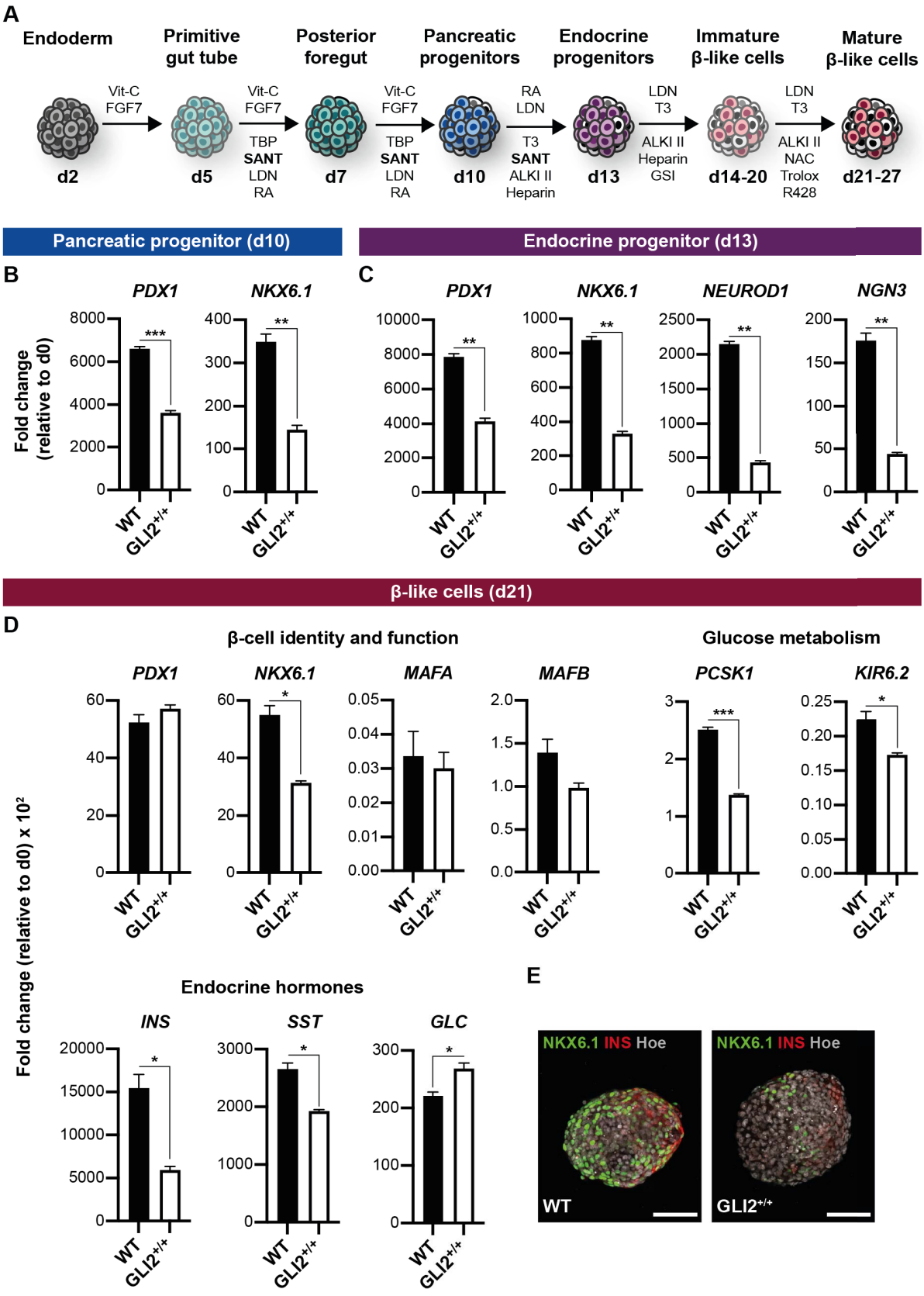

Fig. 24: Modulation of the SHH pathway supports differentiation of GLI2$^{+/+}$ iXM001 iPSCs into β-like cells. (**A**) Schematic diagram of the protocol used to differentiate human iPSCs into β-like cells. (**B**), (**C**) and (**D**) RT-qPCR analyses of indicated stage-specific markers. Values are normalized to GAPDH and relative to day 0. Error bars represented as SEM. *p < 0.05, **p < 0.01, ***p < 0.001. (**E**) Representative whole-mount immunostaining for NKX6.1 and INSULIN of WT- and GLI2$^{+/+}$ iXM001 iPSC-derived β-like cell cluster at day 27. Scale bars, 25 µm.

expression of essential genes for β-cell identity and function (Fig. 24D). Even though the GLI2$^{+/+}$-derived β-like cells expressed some key β-cell markers, immunostaining analysis showed that the number of NKX6.1- and INSULIN-double-positive β-like cells was markedly lower compared to WT control cell clusters (Fig. 24E). Interestingly, the expression of *GLUCAGON* was upregulated in GLI2$^{+/+}$-derived β-like cells. Further investigations are required to address if this is concordant with an increase in the percentage of double positive INSULIN and GLUCAGON polyhormonal cells. Moreover, the functionality of the GLI2$^{+/+}$-derived β-like cells needs to be assessed.

In summary, these findings indicate that modulation of the SHH signalling pathway helps the directed differentiation of GLI2$^{+/+}$ iXM001 iPSCs into β-like cells. Although under the Rezania et al. differentiation conditions, the GLI2$^{+/+}$ iXM001 iPSC-derived cells could progress further along differentiation, the obtained endocrine progenitor cells displayed lower expression of NKX6.1 and PDX1 compared to the controls.

4.5.7 RNA-seq profiling of endoderm and endocrine progenitor cells obtained from GLI2$^{+/-}$ iPSCs

To further characterize the impaired differentiation of GLI2$^{+/-}$ iPSCs, I performed bulk RNA-seq of mutant and control cells at subsequent stages of differentiation. Specifically, I extracted total RNA from WT and GLI2$^{+/-}$ iPSCs at three distinct time points (iPSC [day 0], endoderm [day 5] and endocrine progenitor stage [day 14]) from two independent differentiation experiments. Library construction and sequencing were performed by GENEWIZ.

For each sample a comparable number of high-quality raw reads was obtained and used to estimate the relative abundance of transcripts. The latter was calculated by estimating the fragments per kilobase of exon per million mapped fragments (FPKM). On average 33468680.08 ± 4172290.022 annotated unique transcript reads per sample were detected. To compile a list of differentially expressed genes, transcripts with an absolute $\log_2$ (fold change) in gene expression greater or inferior than 1 were taken into consideration. Based on this criterion, various genes were found differentially expressed between distinct samples. First, I compared WT iPSCs at pluripotency stage to endocrine progenitor cell (day 14) stage. As expected, the pancreatic gene program (*FOXA2*, *SOX9*, *PDX1* and *NKX6.1*) was upregulated, while the pluripotency program was downregulated (*NANOG*, *SOX2* and *OCT4*) (Fig. 25A). Furthermore, the transcript profile of WT and GLI2$^{+/-}$-derived endoderm cells (EN) was comparable with only 48 genes found to be significantly de-regulated in GLI2$^{+/-}$ cells at day 5

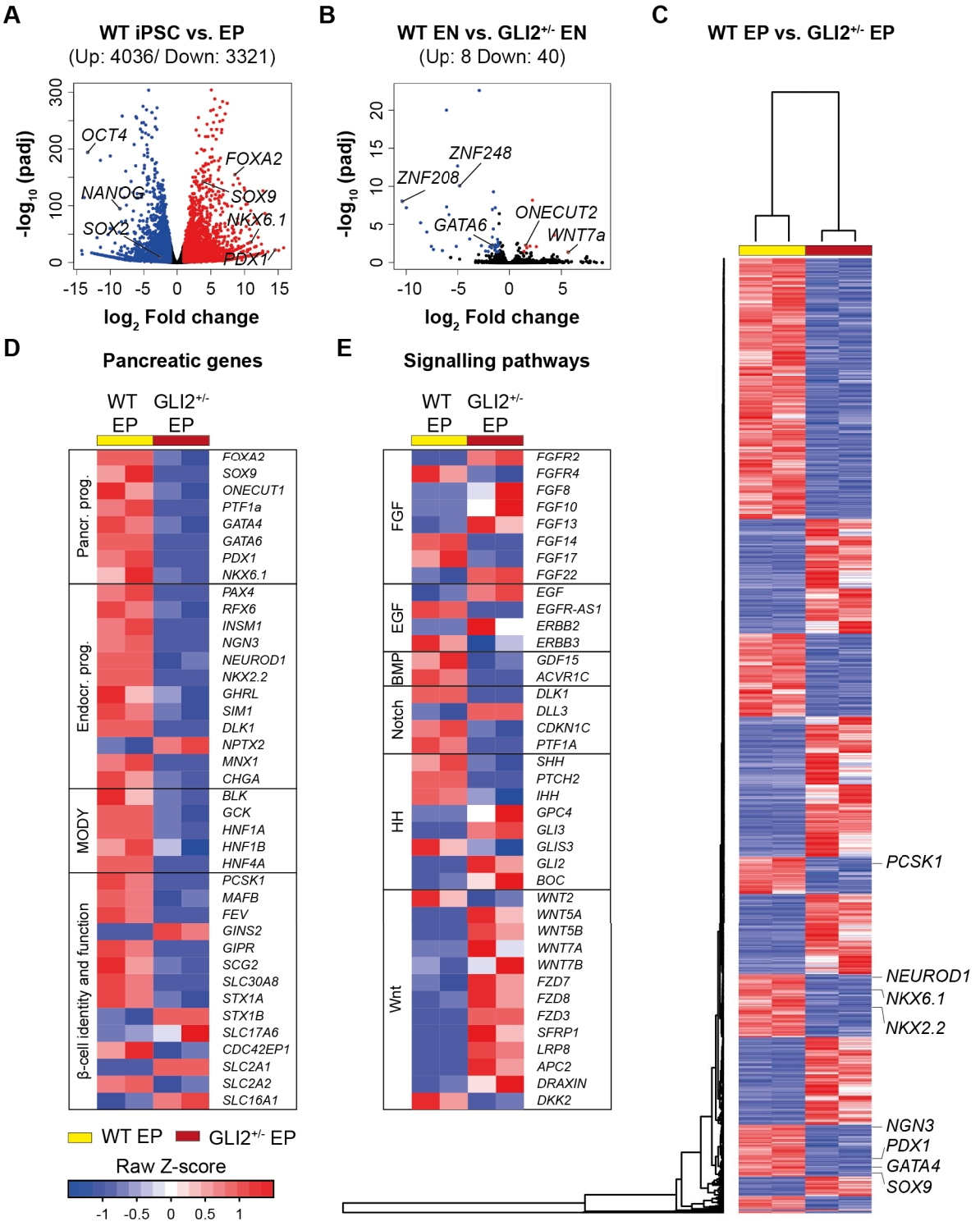

Fig. 25: Whole-transcriptome analysis of GLI2$^{+/-}$-derived cells during differentiation into endocrine progenitor cells. (**A**) and (**B**) Volcano plots visualizing the global transcriptional change across the groups compared. Each data point in the plot represents a gene. Genes with an adjusted p-value less than 0.05 and a log2 fold change greater than 1 are indicated by red dots. These represent up-regulated genes. Genes with an adjusted p-value less than 0.05 and a log2 fold change less than -1 are indicated by blue dots. These represent down-regulated genes. (**C**) Heatmap and hierarchical clustering of differentially expressed genes between patient-like GLI2$^{+/-}$- and WT-derived endocrine progenitors (EPs). (**D**) and (**E**) Heatmap of selected differentially expressed genes between patient-like GLI2$^{+/-}$- and WT-derived EPs. Boxes highlight genes belonging to the indicated categories. Colours represent high (red) or low (blue) expression values based on Z-score normalized to FPKM values for each gene.

(Fig. 25B). Apart from *GATA6* (downregulated) and *ONECUT2* (upregulated), which play a role during pancreas development, the majority of the dysregulated transcripts mapped to zinc finger (ZNF) genes (e.g. ZNF248 or ZNF208) of unknown function (Fig. 25B). By contrast, at endocrine progenitor (EP) stage 928 genes were upregulated and 1070 downregulated in mutant *versus* control cells. Among the most significantly downregulated genes in the mutant cells were genes crucial for endocrine differentiation, including *PDX1*, *NKX6.1*, *NGN3*, *NEUROD1*, *NKX2.2* (Fig. 25C). Consistent with the previously described phenotype, a closer look into the RNA-seq data revealed a general downregulation of genes involved in pancreatic development (Fig. 25D). In particular, transcripts coding for pancreatic progenitor markers, such as *FOXA2*, *SOX9*, *ONECUT1*, *PTF1a*, *GATA4*, *PDX1* and *NKX6.1*, were significantly downregulated in mutant EP cells compared to controls. Also, transcripts of genes whose expression is crucial during endocrine differentiation, such as *NGN3*, *NEUROD1*, *NKX2.2* and *INSM1,* were reduced in mutant EP cells. Interestingly, the expression of *NPTX2* was increased in GLI2$^{+/-}$ EP cells. *NPTX2* plays a role during excitatory synapse formation and was also reported to be enriched in endocrine populations displaying a β-like cell profile (Fig. 25D) [20]. In addition, MODY genes, namely *BLK*, *GCK*, *HNF1A*, *HNF1B* and *HNF4A* were downregulated in mutant EP cells. Furthermore, genes important for β-cell identity (e.g. *MAFB*, *FEV*), metabolic sensing (e.g. *SCG2* and *SLC30A8*), exocytosis (e.g. *STX1A*, *CDC42EP1*), insulin processing (e.g. *PCSK1*) and glucose sensing (e.g. *SLC2A2*, *GCK*) were less abundant in GLI2$^{+/-}$-derived EP cells. A subset of the above mentioned differentially expressed genes was confirmed by RT-qPCR and in previous analyses (Fig. 26A, 21 and 22).

Cell fate commitment is controlled by a variety of signalling pathways. Therefore, I performed gene ontology (GO) analysis to identify biological processes associated with the observed phenotype in GLI2$^{+/-}$ EPs. GO analysis showed that top downregulated categories were enriched with factors involved in developmental processes, with the endocrine pancreas being one of the top affected lineages (Fig. 26B). Moreover, regulation of insulin secretion, cell fate commitment and glucose homeostasis GO terms were enriched among the genes downregulated in the GLI2$^{+/-}$ EP cells. KEGG pathway analysis showed that GLI2$^{+/-}$-differentially expressed genes were mainly involved in MODY, insulin secretion, pancreatic secretion, axon guidance and T2D categories (Fig. 26C). These results underscore that the putative pathogenic patient variant c.C4661T impairs endocrine pancreas development and, consequently, contributes to the disease development and/or progression of diabetes.

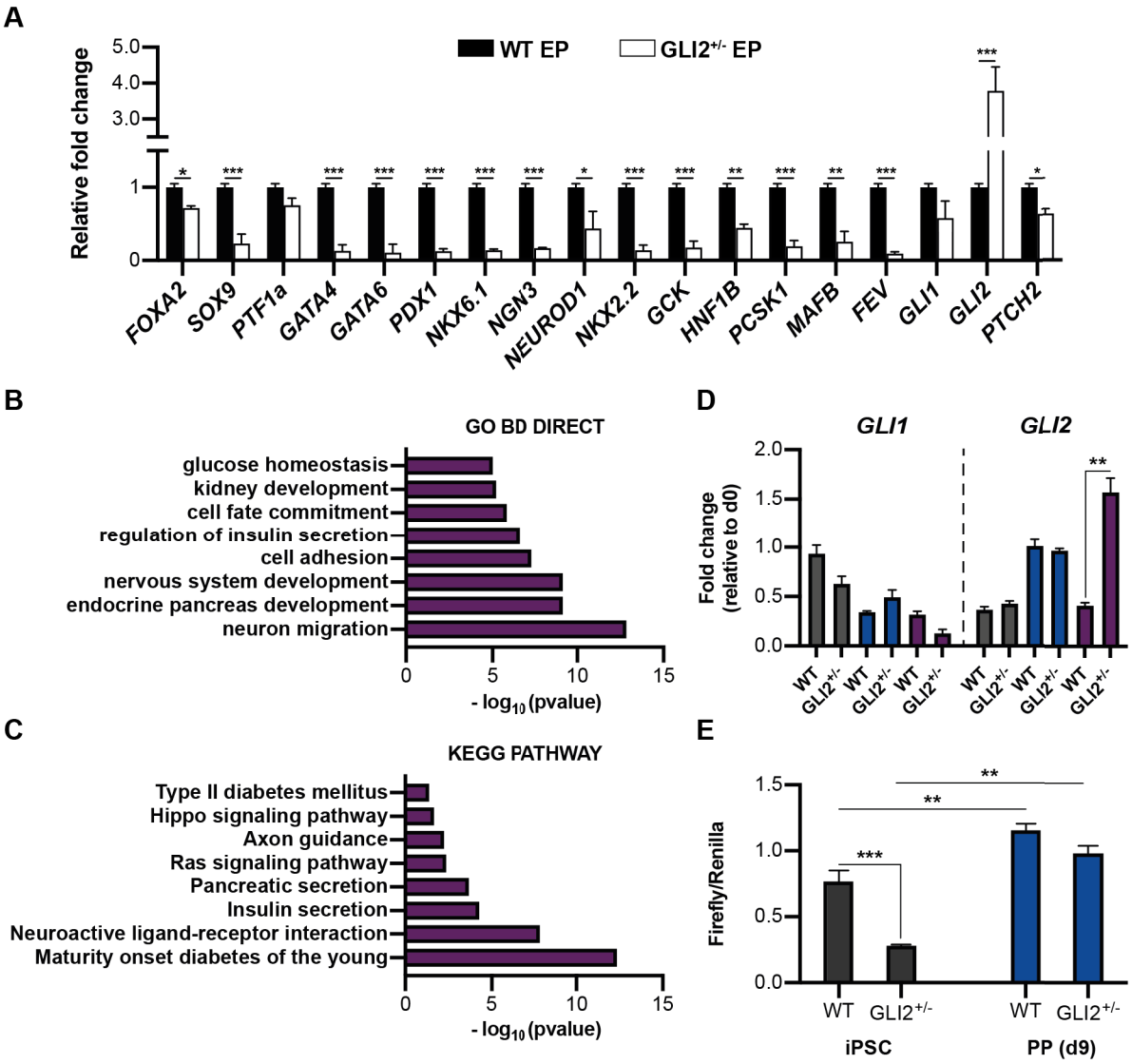

Fig. 26: RNA-Seq profile of GLI2$^{+/-}$-derived endocrine progenitor cells. (**A**) RT-qPCR validation of a subset of differentially expressed genes in GLI2$^{+/-}$- and WT-derived endocrine progenitor cells. (**B**) and (**C**) Gene ontology enrichment analysis of differentially expressed genes ($p < 0.05$) performed on EP RNA-Seq datasets. (**D**) RT-qPCR analysis of GLI1 and GLI2 during differentiation into endoderm (grey), pancreatic (blue) and endocrine progenitors (purple) from WT or GLI2$^{+/-}$ iPSCs, respectively. (**E**) GLI luciferase assay in WT and GLI2$^{+/-}$ iPSCs and their derivatives at pancreatic progenitor (PP) stage, respectively. At PP stage, cell clusters were dissociated and transfected the next day with WT luciferase reporter plasmid and the internal control plasmid (Renilla). Results were normalized for transfection efficiency using Renilla luciferase and are represented as Firefly/Renilla activity ratio. The experiment was performed in triplicates and repeated independently two times. Error bars represent SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

To start to investigate the molecular pathways that are dysregulated during pancreatic differentiation of GLI2$^{+/-}$ iPSCs, I further examined the expression of several pathway components at endocrine progenitor stage (Fig. 25E). RNA-seq analysis revealed modulated expression of genes involved in FGF, EGF, Notch and Wnt signalling pathway (Fig. 25E). Of note, several Wnt ligands (e.g. *WNT5A*, *WNT5B*, *WNT7B*) and Wnt receptors (e.g. *FZD3*, *FZD7*, *FZD8*) were upregulated in GLI2$^{+/-}$ EP cells. Additionally, the Wnt inhibitor *DKK2* was

downregulated. This could be of further interest because previous studies in mice have demonstrated that overexpression of Wnt components results in severe pancreatic defects, including pancreatic agenesis and pancreatic hypoplasia [192]. Furthermore, genes of the HH signalling pathway were differentially expressed between WT and GLI2$^{+/-}$ EP cells (Fig. 26E). Notably, the expression of *GLI2* was strikingly induced in mutant EP cells (Fig. 25E, Fig. 26A and D). Contrary, *GLI1* was not differentially expressed at endocrine progenitor stage between mutant and WT cells (Fig. 26A and D). To assess if the activation of the SHH pathway is impaired in GLI2$^{+/-}$ cells, I performed a GLI-mediated transcriptional activation assay in WT and GLI2$^{+/-}$ iPSCs and their derivatives at pancreatic progenitor (PP) stage, as previously described (Fig. 10). The PP stage was selected because it coincides with the time when both *PDX1* and *NKX6.1* was altered in mutant cells (see Fig. 21C). The GLI-induced luciferase activity of the reporter in transfected HEK293T cells was reduced in the presence of GLI2 p.P1554L (c.C4661T) variant (see Fig. 10). Consistently, in GLI2$^{+/-}$ iPSCs the endogenous transcriptional activation of the GLI-responsive luciferase reporter was reduced in comparison to WT iPSCs (Fig. 26E). However, this did not impair the transcriptional activity at PP stage (Fig. 26E). This could be due to the increased levels of GLI2 observed at endocrine progenitor stage that rescue transcriptional activation of HH. To further investigate the activity of the HH pathway during differentiation, the assay needs to be repeated at endocrine progenitor stage. Moreover, ongoing studies are examining the localization of the WT and mutant GLI2 proteins. Together these studies will shed some light on the potential mechanism of GLI2$^{+/-}$.

4.6 Human iPSC differentiation platform can be used to study extrinsic factors during β-cell differentiation

Over the past years, considerable progress has been achieved in unravelling key regulators of pancreatic development. This knowledge has further improved the differentiation efficiency of pluripotent stem cells toward pancreatic β-cells by manipulating signalling pathways in a stepwise manner to recapitulate pancreatic development *in vitro*. Moreover, large-scale screenings have identified novel small compounds that improve the generation of pancreatic progenitors. Nevertheless, the discoveries of extrinsic and intrinsic factors that regulate cell fate decisions or enhance cell survival are of great interest for both basic research and clinical application.

4.6.1 HC toxin, an HDAC inhibitor, stimulates the expression of β-cell markers and the function of iPSC-derived β-like cells

Pancreas development initiates with the expression of the TF PDX1 within the pancreatic endoderm where it is required for the specification of all endocrine cell types. Subsequently, PDX1 becomes restricted to the β-cell lineage, where it plays a crucial role in β-cell function. This pivotal role of PDX1 at various stages of pancreas development makes it an attractive target to enhance pancreatic β-cell differentiation and increase β-cell function. Therefore, the lab of Prof. Dr. Didier Y. Stainier (Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany) generated a novel zebrafish reporter to screen over 8000 small molecules for modulators of *pdx1* expression. Besides known modulators of *pdx1* expression, they identified four novel hit compounds, including the HDAC inhibitor HC toxin (HC).

To validate and determine whether the induction of *pdx1* is conserved across species, I used the previously described iPSC differentiation platform (Fig. 27) and exposed the cells to HC during the last step of differentiation. Specifically, I treated iPSCs undergoing differentiation with HC for 24 hrs. at day 18 and, subsequently, analysed the cells by RT-qPCR and immunofluorescence at day 21 (Fig. 27A). Strikingly, I observed an increase in the expression levels of key β-cell TFs, such as *PDX1*, *NKX6.1*, *NKX2.2*, *NEUROD1*, as well as *INSULIN*, after exposure to HC, suggesting a conserved role of HC also in human β-cell differentiation (Fig. 27B). In addition, the transcript levels of the mature human β-cell transcription MAFB was robustly induced in cells treated with HC (Fig. 27B). Consistently,

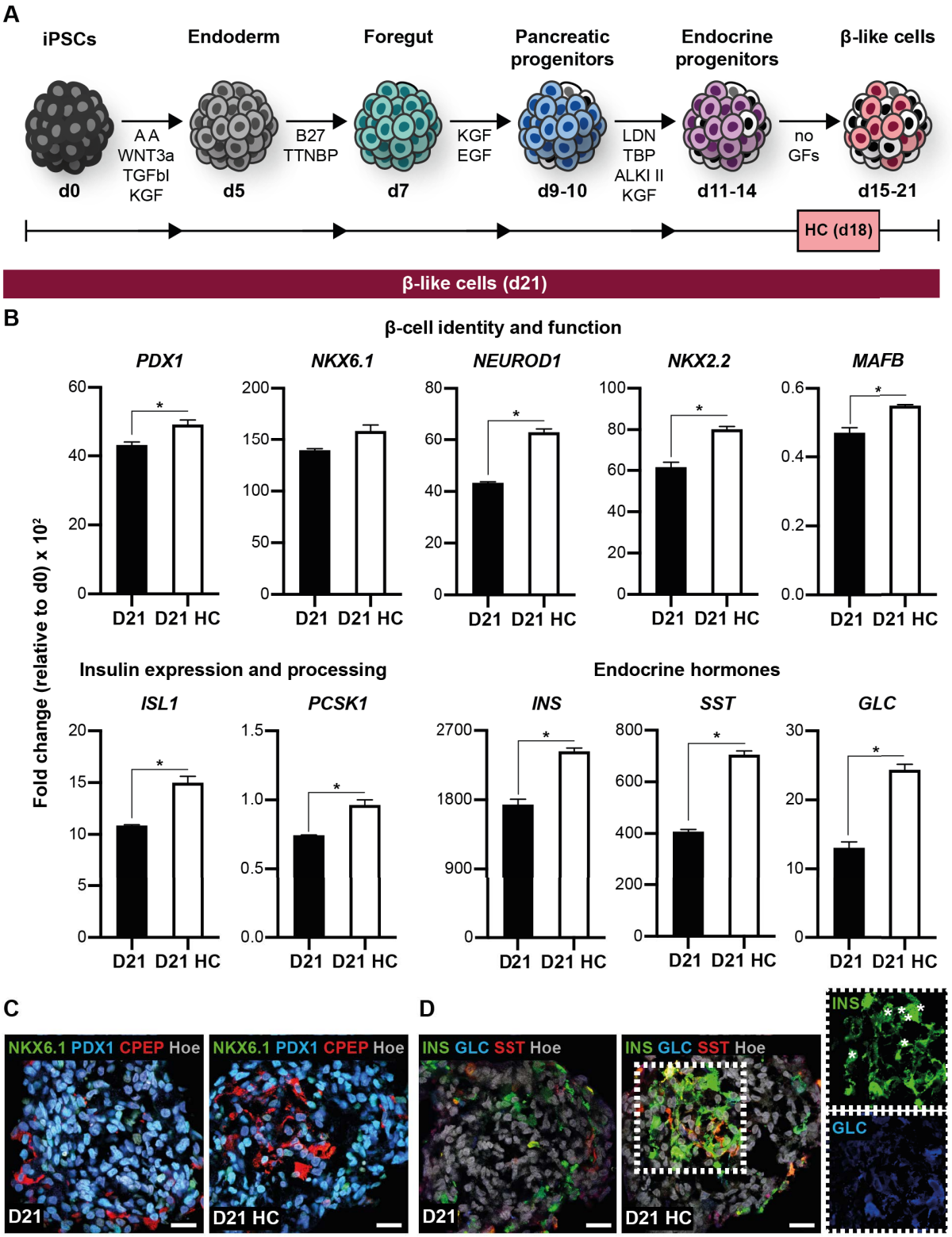

Fig. 27: HC toxin induces the expression of β-cell differentiation markers *in vitro*. (**A**) Schematic of the differentiation protocol of iPSCs into β-like cells. Cells were treated with HC toxin for 24 hrs. at day (d) 18 of differentiation. (**B**) RT-qPCR analysis of selected gene expression levels at D21 (differentiated cells) and D21 HC (differentiated cells following treatment with HC toxin) relative to undifferentiated cells (D0). *p < 0.05. Error bars represent SEM. (**C**) and (**D**) Immunostaining of differentiated clusters at day 21 for NKX6.1, PDX1, c-peptide (CPEP) and insulin (INS), somatostatin (SST), and glucagon (GCG), respectively. Hoechst 33342 (Hoe) was used as nuclear counterstain (control n = 12; HC Toxin n = 25). Asterisks mark monohormonal cells. Scale bars, 20 µm.

immunofluorescence analysis revealed nuclear localization of TFs critical for β-cell function (PDX1 and NKX6.1) and their co-expression with C-PEPTIDE (Fig. 27C). The induced β-like cells were positive for INSULIN and the other endocrine hormones, like GLUCAGON and SOMATOSTATIN (Fig. 27D), but I detected only few polyhormonal cells (3.12 ± 0.4 %) in the differentiated clusters. Importantly, in the presence of HC the percentage of polyhormonal cells (INSULIN$^+$ and GLUCAGON$^+$) was further reduced (2.67 ± 0.25 %). Taken together, these results indicate that HDAC inhibition enhances differentiation of β-cells from human pluripotent cells. This study was recently published [419].

4.6.2 SLIT3 supports the generation of human endocrine progenitors

Recent studies in the Spagnoli lab have identified a set of novel mesenchymal factors, which support the establishment of pancreatic endoderm progenitors and increase the generation of insulin$^+$ cells in mice (Cozzitorto et al., under revision). Among these factors, the highly conserved axon guidance signalling molecule SLIT3 was identified. Previous findings underscored the role of the Robo/Slit pathway in various aspects of pancreas organogenesis as well as pancreatic cancer [420–422]. Moreover, it was shown that Robo1 and Robo2 receptors are expressed in pancreatic progenitors starting from fate specification onwards [423].

To study, whether SLIT ligands enhance the differentiation of human iPSCs into endocrine progenitors and β-like cells, human SLIT3 recombinant protein was added to the differentiation cocktail for 48 hrs. during the transition from endoderm to foregut stage (Fig. 28A). At morphological level, no apparent differences were noted between control and SLIT3-treated cell clusters, ruling out potential cytotoxicity of the tested SLIT3 concentration. The differentiation of iXM001 iPSCs was examined by RT-qPCR analysis of key endoderm (*FOXA2, SOX17*), foregut (*PDX1, FOXA2*), pancreatic progenitor (*PDX1, NKX6.1*), endocrine progenitor (*NEUROD1, NGN3*) and β-like cell (*INSULIN*) marker genes. Importantly, significant differences became detectable at the endocrine progenitor stage (day 14) between untreated control and SLIT-treated clusters (Fig. 28B). In particular, the TF NEUROD1, which is known to promote endocrine differentiation and maintain β-cell function, was upregulated in SLIT3-treated cell clusters at day 14. Moreover, the expression of *PAX6* and *INSULIN* was significantly induced after SLIT3-treatment. In detail, *INSULIN* expression level was about 1.3-fold in treated cells than in control clusters and remained higher in differentiated β-like cells at day 21 (Fig. 28A). Consistently, immunohistochemistry showed a significantly increase in cells double positive for NKX6.1 and INSULIN in SLIT3-treated cell clusters at endocrine progenitor stage (Fig. 28C). Indeed, cell counting revealed an increase in double positive cells for NKX6.1

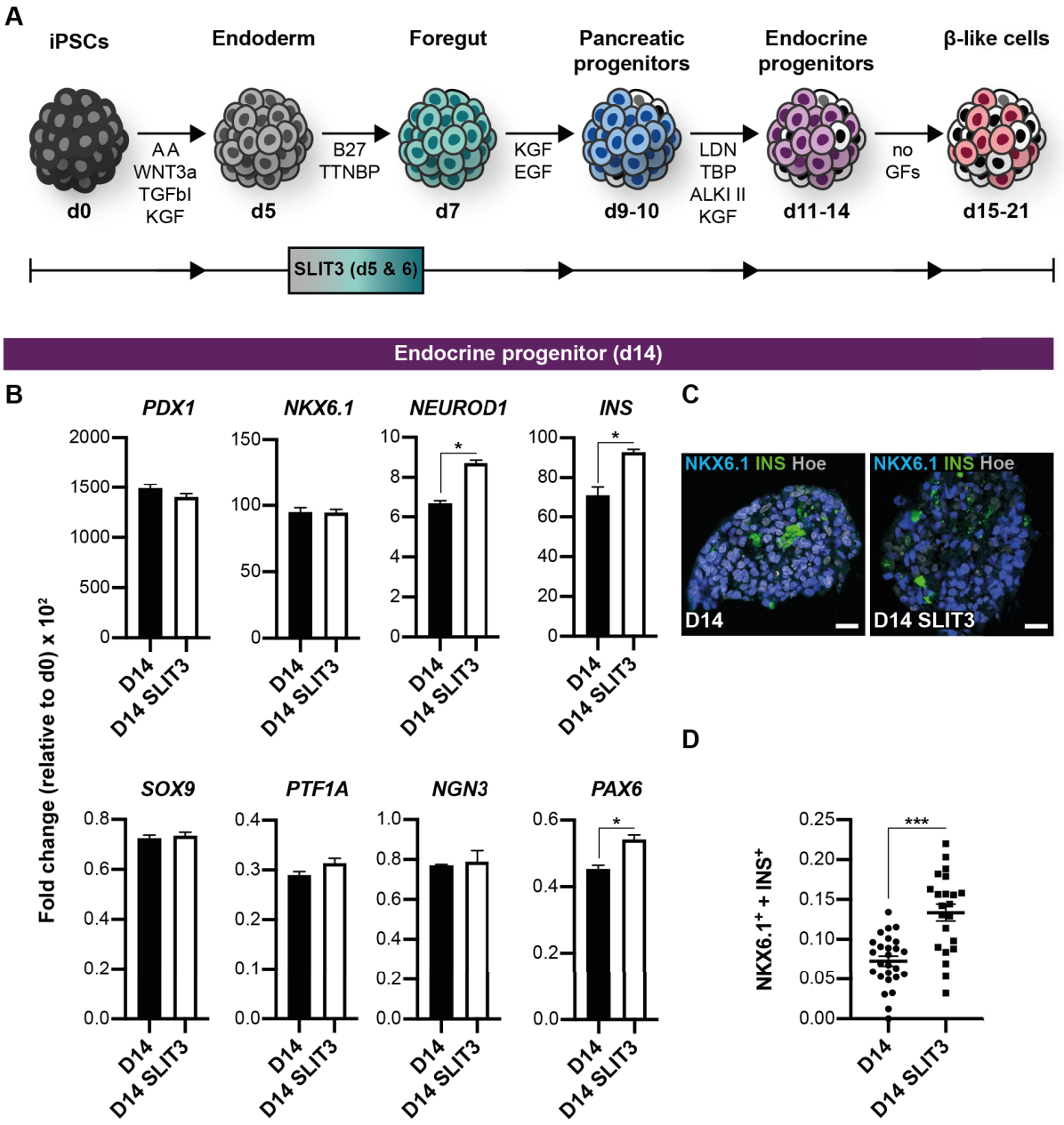

Fig. 28: Functional conservation of pro-endocrine mesenchymal signal factors. (**A**) Schematic of the differentiation protocol of iPSCs into β-like cells. Cells were treated with SLIT3 for 48 hrs. at day (d) 5 of differentiation. (**B**) RT-qPCR analysis of selected gene transcripts in differentiated cells at D14 cultured with SLIT3 or untreated. Data are represented as fold change relative to undifferentiated cells (d0). Values shown are mean ± SEM. n=3. *p < 0.05. (**C**) Representative IF images for NKX6.1 and INSULIN (INS) on differentiated clusters at D14. Hoechst (Hoe) was used as nuclear counterstain. Scale bars, 20 µm. (**D**) Scatter plot showing significant increase of NKX6.1- and INSULIN-double positive cells upon treatment with SLIT3. The % of NKX6.1⁺/INSULIN⁺ cells was quantified and normalized to the total number of cells contained in each cluster. n=3 control untreated cell cultures, n=3 SLIT3-treated cell cultures (n represents biologically independent differentiation experiments). ***p < 0.001.

and INSULIN by about 83% after SLIT3 treatment (Fig. 28D). Notably, the NKX6.1- and INSULIN-double positive population has been recently characterized for being more closely related to endogenous human β-cells than the NKX6.1-negative one. In contrast the

expression of early pancreas progenitor markers, like *SOX9*, *PTF1A* and *NGN3*, remained unchanged after SLIT3 treatment. The expression of *PDX1*, *NKX6.1*, *NEUROD1* and *INSULIN* remained induced in SLIT3-treated β-like cells at day 21. Moreover, the human β-cell TF MAFB was robustly expressed in the treated β-like cells, as well as, other genes essential for human β-cell functionality, such as the *GCK1* and *KIR6.2* (Fig. 29A). Although the expression of *SOMATOSTATIN* and *GLUCAGON* was induced, this did not result in an increase of polyhormonal cells. Actually, the percentage of INSULIN- and GLUCAGON-double positive cells in differentiated clusters was further reduced (2.67 ± 0.25 %) by SLIT3 when compared to the number of polyhormonal cells obtained in the control clusters (3.12 ± 0.4 %). Finally, preliminary results suggest that SLIT3 supports mature functionality of derived β-like cells as judged by improved GSIS (Fig. 29B).

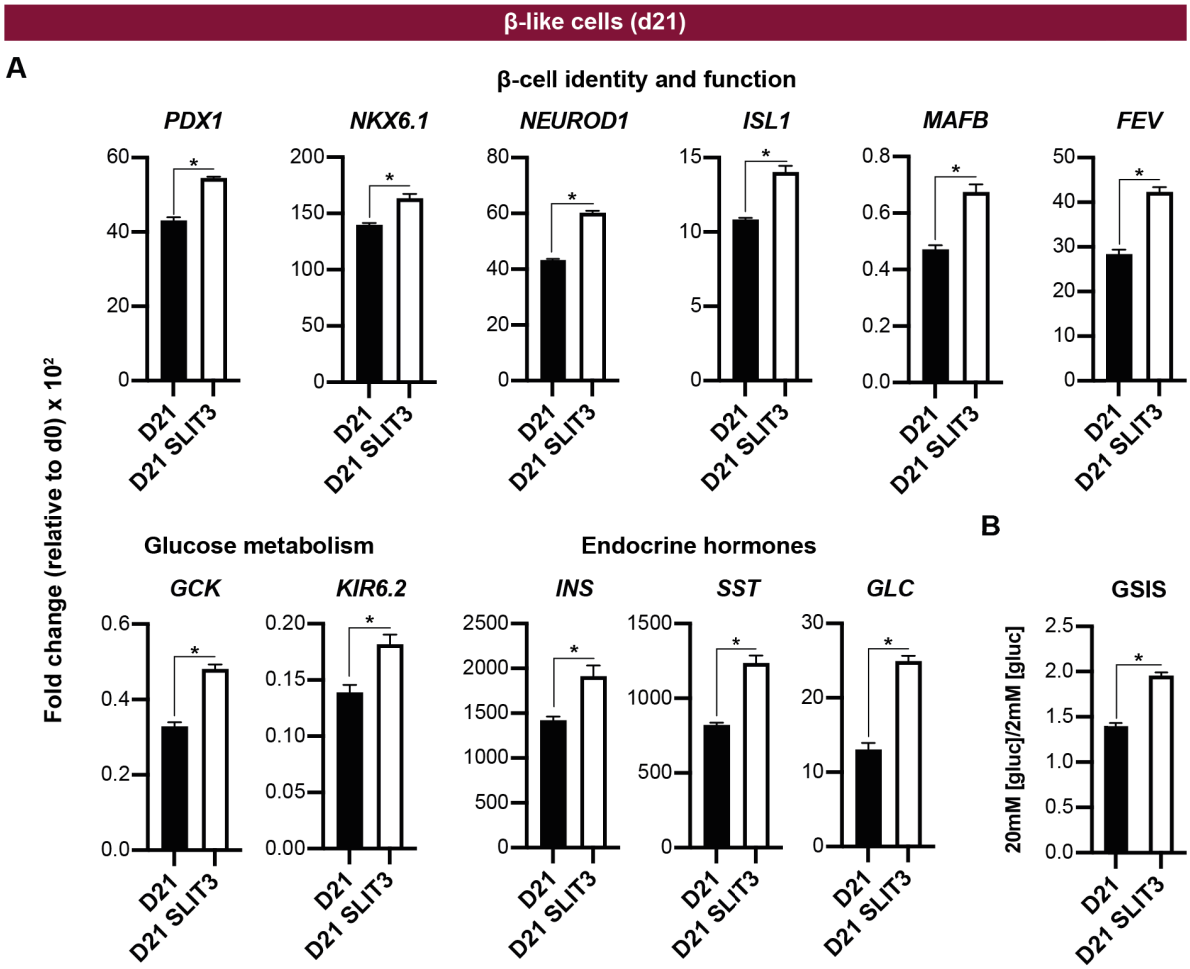

Fig. 29: Treatment with SLIT3 supports differentiation into iPSC derived β-like cells. (**A**) RT-qPCR analysis of selected gene transcripts in differentiated cells at D21 cultured with SLIT3 or untreated. Data are represented as fold change relative to undifferentiated cells (d0). Values shown are mean ± SEM. n=3. *p < 0.05. (**B**) GSIS assay of untreated and SLIT3-treated β-like cells at day 21. Y-axis indicates ratio of insulin secreted in low glucose conditions to that secreted in high glucose conditions. One experiment is shown. *p < 0.05.

In conclusion, these results indicate that stimulation of SLIT guidance pathway enhances endocrine differentiation from human pluripotent cells, suggesting functional conservation with the mouse. Further investigations are ongoing to define in more detail the specific window of action (e.g. treatment at different time points), dose response, and impact on β-cell functionality. These results are included in a manuscript that is currently under revision (Cozzitorto et al., under 2nd revision).

5 DISCUSSION

5.1 Identifying putative new candidate disease-genes for diabetes in patient cohorts

Diabetes is a group of metabolic diseases characterized by hyperglycaemia and still a leading cause of mortality and morbidity worldwide. Currently, there is no cure for diabetes and therapeutic options are limited [13,215–217,424]. Contrary to the more common form of diabetes, T1D and T2D, monogenic diabetes arises from rare mutations in one single gene [64–66,375]. Thus, they represent invaluable models for identifying new key targets of β-cell dysfunction as well as development in a human context [425]. However, monogenic diabetes, like MODY, is clinically and genetically heterogeneous, as molecular dysfunction of each presumed causal gene manifests in a distinct clinical phenotype [257,375,426]. Thus, an accurate molecular diagnosis and cautious extrapolation of sequence data are critical for effective disease treatment. Although, about 3% of diabetic patients involve single-gene mutations (Mendelian), the majority of patients with monogenic diabetes are unrecognized and misdiagnoses as T1D or T2D [148,427,428]. However, the benefit of genetic testing has been shown in patients with monogenic diabetes caused by a mutation in *HNF1A*, *HNF4A* or *KCNJ11,* respectively. All these patients are sensitive to sulfonylureas, which greatly improves glycaemic control [256]. Therefore, identification of novel disease-related genes may not only provide new insight in the pathogenesis of diabetes but also open opportunities to develop novel diabetic drugs to improve treatment and disease prognosis.

To date, over 30 genes have been linked to monogenic diabetes and the list continues to be expanded thanks to advances in genomic research [64,65]. Still in numerous families with non-autoimmune diabetes the genetic defects remain obscure, hindering our understanding of the biology of diabetes. Moreover, even in the post-next-generation sequencing era, the identification and annotation of novel pathogenic variants for Mendelian and other postulated monogenic diseases are challenging. There are also examples of disease-causing variants that fail to cause the respective disease in a subset of individuals who carry them. This phenomenon is known as reduced or incomplete penetrance [429]. Indeed, unlike previously assumed incomplete penetrance is not uncommon. For instance, heterozygous RFX6 protein truncating variants have a reduced penetrance of diabetes

compared to common HNF1A and HNF4A-MODY mutations [68,430]. Incomplete penetrance could be attributed to several factors, such as age, gender, epigenetic modification, or environmental modifiers [128,431]. In our unique cohort we identified several *de novo* putative pathogenic variants in *HDAC4* (c.A680G and c.G700A) and *GL1* (c.G1414T), which were not present in the patient's parents or other healthy controls, as well as an inheritable variant in GLI2 (c.C4661T). The latter one did not completely segregate in the family. While both index children displayed insulin-dependent diabetes, the parents showed a milder (T2D, OADs) or asymptomatic clinical phenotype.

Beside the penetrance issue, our ability to identify disease-relevant sequence variants by far exceeds our capacity to determine their functional consequences. To overcome this obstacle, I combined clinical phenotypic data, bioinformatics prediction and initial molecular characterization to assign pathogenicity with confidence to individual variants. Through next-generation sequencing of families with puberty-onset diabetes and Sanger sequencing, validation, missense mutations in the *HDAC4*, *GLI1* and *GLI2* gene were identified as possibly etiological variant for the respective families. Among the variants, two variants in GLI2, namely c.G3099C and c.C4661T were novel, while the others in *HDAC4*, *GLI1* and *GLI2* have been deposited in databases (ExAC, NCBI dbSNP). Nevertheless, all these variants are rare (MAF < 0.01). In addition, *in silico* predictions indicated that the majority of these variants may alter the normal structure and function of the respective proteins and represent therefore potential causes of the diabetic phenotype. Furthermore, initial gain-of-function experiments in *Xenopus laevis* and β-cell lines helped to define a priority list of the identified gene variants for iPSC disease modelling. The *Xenopus* is a remarkable *in vivo* model system to study early events in development, which are highly conserved across vertebrate species [406–408]. Also, previous studies showed that the *Xenopus* model system can successfully be used to interrogate candidate gene variants in diabetes. Simaite et al. used morpholino-mediated knockdown experiments in *Xenopus* to characterize a frame-shift mutation in *PCBD1* (pterin-4 α-carbinolamine dehydratase/ dimerization cofactor of hepatocyte nuclear factor 1 α) found in a cohort of individuals with early-onset diabetes (antibody-negative for T1D) [432]. Similarly, to define the role of RFX6 in early endoderm development a previous study performed morpholino knockdown of rfx6 in *Xenopus* embryos. Mutations in *RFX6* are associated with NDM and knockdown of this gene in *Xenopus* caused a loss of pancreas marker expression. Thus, to characterize the role of HDAC4 during pancreas development I performed gain-of-function studies in *Xenopus* embryos. These studies suggested that deregulation of HDAC4 impairs β-cell development but where somehow inconclusive regarding the effect of the identified *HDAC4* variants. HDAC4 is a member of the ubiquitously important family of class IIa HDACs and potential functional redundancy among members of the HDAC family might have obscured a phenotype.

Taking together, this represents a fast and cost-effective *in vivo* platform to systematically validate and characterize genes identified in cohorts of families with rare diabetes types of unknown pathogenesis. Coupling this further with human cell models, like iPSCs, allows to functionally dissect the role of the identified variants.

5.2 CRISPR-Cas9 engineered lines represent a novel human *in vitro* iPSC model to study the role of *HDAC4*, *GLI1* and *GLI2* during pancreatic development

In this study, I established human iPSC-based models to investigate the molecular mechanisms by which *HDAC4*, *GLI1* and *GLI2* regulate both β-cell development and function. To establish a platform that enables to interrogate multiple putative diabetes-linked variants in parallel, I used a previously developed gene-editing platform in human iPSCs allowing DOX-regulated expression of Cas9 (iCRISPR) [331,433,434]. Employing this system, simple transfection of the sgRNA and repair template in DOX-treated iPSCs, allowed efficient generation of patient-like iPSC lines through HDR. These patient-like iPSCs together with their WT counterparts were subsequently differentiated towards β-like cells and characterized at distinct developmental stages. Overall, the iCRISPR system in combination with directed differentiation is a very powerful tool for disease modelling, avoiding confounding effects related to species differences between mouse and humans.

Based on the available patient information, initial *in silico* and functional analysis, I assigned priority to the patient variant HDAC4 c.G700A, GLI1 c.C119G and GLI2 c.C4661T for iPSC-based disease modelling. Using the iCRISPR approach, I successfully engineered heterozygous patient-like iPSC lines for *GLI1* c.C119G and *GLI2* c.C4661T, while no patient-like iPSC clone carrying mutations in the *HDAC4* gene could be established. Although multiple attempts of CRISPR-Cas9 gene editing were performed with distinct sgRNAs for *HDAC4* mutant variants, only clones harbouring indel mutations were generated, suggesting that the CRISPR-Cas9 cutting efficiency was not impaired. Moreover, the ssODNs also contained a silent mutation in the PAM sequence to prevent re-cutting by Cas9. By contrast, these results indicate that the precise genome editing in *HDAC4* was hindered by the low efficiency of HDR. In general, HDR competes with NHEJ and is typically restricted to the S and G2 phases of the cell cycle [435]. Recent advances in the CRISPR genome editing system have improved the HDR frequency. For example, Jayavaradhan and colleagues fused a dominant-negative mutant of 53BP1, DN1S, to Cas9 nucleases [333]. The resulting Cas9-DN1S fusion protein significantly blocks NHEJ events at Cas9 cutting sites and thus promotes HDR. Alternative,

strategies to improve the efficiency for HDCR-induced point mutation in human iPSCs have also been developed and do not require a modification of the Cas9 enzyme [436,437]. For instance, it was shown that the 53BP1 inhibitor, i53, increases HDR and thus CRISPR-Cas9 genome editing efficiency [438]. 53BP1 is a key regulator of DBS repair pathway choice in eukaryotic cells and suppresses end resection thereby favouring NHEJ over HDR. Thus, I added a plasmid i53 during sgRNA and ssODN transfection. Nevertheless, this did not support the establishment of a patient-like HDAC4 iPSC line and additional improvements may be required. However, the mutation might also be lethal in iPSCs, which might explain the absence of clones with the desired genotype. Notably, previous studies showed that inhibition of HDACs modulates pluripotency and interferes with early differentiation steps of ESCs [439]

In a second approach, to study the biological function of HDAC4, the CRISPRi system was applied, which is based on a DOX-inducible deactivated Cas9 fused to a KRAB repression domain. This allows specific and reversible knockdown of gene expression in iPSCs and derivatives [338,440]. Once the knockdown of *HDAC4* is confirmed at protein level, the established iPSC knockdown line will be used to dissect the function of HDAC4 during different stages of β-cell development.

Human disease-linked mutations in *GLI1* are very rare. So far only two missense mutations are listed in the HGMD being associated with bicuspid aortic valve or inflammatory bowel disease [389–392]. Importantly, GLI1 has been associated with pancreatic cancer [441,442]. Pancreatic adenocarcinoma is one of the most aggressive forms of cancer, which develops through a multistep carcinogenesis starting with oncogenic mutation of the *KRAS* gene. Dysregulation of GLI1 leads to the expression of transcriptional target genes of GLI1 that govern hallmark of malignant properties [441,443–445]. In mice, induction of a dominant-negative form of Gli1 reduces KRAS-driven tumorigenesis in the pancreas. Moreover, studies in mice highlighted that deregulated HH signalling has profound effects on the developing endocrine cells within the pancreas [173,446]. Nevertheless, the role of GLI1 during β-cell development and function remains elusive. Here we identified a novel putative pathogenic variant in *GLI1* located between the SUFU interaction site and the DNA-binding domain that comprises five tandem C2H2 zinc-fingers. Using the CRISPR-Cas9 system I inserted the c.C119G point mutation into the *GLI1* gene thereby generating a heterozygous patient-like iPSC line. Although only a single clone was analysed, my results suggest that the putative pathogenic variant c.C199G in *GLI1* does not impair the differentiation into β-cells. Further studies are required to confirm this data at the protein level and to test the glucose-responsiveness of the GLI1$^{+/-}$-derived β-like cells. Finally, rare, protein-altering, and putative pathogenic variants do not necessarily give rise to monogenic diabetes. Instead, they can either contribute to T2D predisposition or just exist as benignant variants in the human genome. For example, this has been shown for certain variants in HNF1A [375,447,448]. In

the present study, I introduced the specific variants into a healthy, non-disease affected WT iPSC line. This approach allows the generation of isogenic iPSC lines with the mutation of interest as the sole variable. Moreover, this avoids any discrepancies in differentiation efficiency due to differences in genetic backgrounds or secondary effects resulting from line-to-line variations [449,450]. The genetic background is defined as the genotype of all genes that might interact with the candidate gene and therefore alter the phenotype [451]. To address if the genetic background of the patient plays a role during diabetes development, it would be interesting to derive iPSCs directly from patients heterozygous for c.C199G in *GLI1* and differentiate them towards the pancreatic lineage. Previous studies have underscored the discrepancies and variabilities in phenotype among different WT iPSC lines. CRISPR-Cas9 could then be employed to establish isogenic controls by correcting the patient mutation. Observed defects in β-cell development can then be attributed to the genetic background of the patient which increases the susceptibility to diabetes. Alternatively, an *in vitro* model may not accurately capture disease progression. Although current differentiation protocols mimic essential *in vivo* developmental events, the use of a cocktail of small molecules and growth factors to drive the process *in vitro* may lack certain signals that patient cells encounter under an *in vivo* setting. One important component that is currently missing in directed differentiation protocols is, for example, the extracellular microenvironment. Of further note, the pathogenicity of c.C119G could also emerge via non-β-cell-autonomous mechanisms. This hypothesis could involve different non-β-cells residing in the endocrine/exocrine pancreas or other organs (e.g. liver, gut) that for example affect insulin secretion through paracrine or endocrine signalling. In this case, an organoid-based model of GLI1$^{+/-}$ could be applied where multiple cell types are derived from GLI1$^{+/-}$ iPSCs. Organoids are 3D structures derived from PSCs, which consist of self-organized organ-specific cell types [83,452].

5.3 The new variant c.C4661T in *GLI2* impairs development of endocrine progenitor cells

In this study I successfully established iPSC lines carrying the patient variant c.C4661T in *GLI2* using CRISPR-Cas9 genome editing. This variant was detected by exome sequencing in a consanguineous family with reduced penetrance of diabetes. The effect of the mutant protein was first examined in different cell lines, including HEK293T and MIN6 cells, and suggested to alter and decrease the activity of the WT protein. Consistent with this, *in silico* analysis predicted that the variant is pathogenic and might thus be the possible disease-causing variant for the family.

Mutations in the human *GLI2* gene have been reported as causative of a spectrum of clinical phenotypes, including holoprosencephaly, postaxial polydactyl and hypopituitarism [395,453]. Moreover, the zinc-finger TF GLI2 is a downstream target of the HH signalling pathway, which in mice plays a role during pancreas organogenesis, β-cell formation and function [173,189,446,454]. Given that the variant is located in the transcriptional activation domain, it is likely that it alters the activity of the HH signalling pathway, thereby hampering pancreas development. Indeed, using pancreatic differentiation of patient-like iPSCs, I demonstrated that the variant c.C4661T in *GLI2* significantly impairs endocrine progenitor development in a dose-dependent manner. This was evident by the reduced number of PDX1 and NKX6.1 double-positive endocrine progenitor cells, as well as the diminished expression of endocrine markers like *NGN3*, *NEUROD1* and *NKX2.2*. In line with this, lack of *NGN3* leads to developmental failure of endocrine cells in mice and humans. Moreover, it was previously published that the expression level of NGN3 is essential for endocrine commitment and its window of expression determines endocrine cell fates [58,60,93,94]. Therefore, the downregulation of *NGN3* observed in patient-like iPSCs might lead to endocrine progenitor pool depletion, resulting in reduced β-cell mass at birth. Based on these results it would be of interest to know if GLI2 variant carrier exhibit a reduction in pancreatic islets or β-cells. Unfortunately, pancreatic images, like magnetic resonance imaging, are not available at this point.

GLI2 encodes for a TF that controls the expression of several genes in the HH pathway. Unlike GLI1, GLI2 and GLI3 can act either as a transcriptional repressor or activator. In the absence of HH ligand, GLI2 and GLI3 are partially truncated at the C-terminus, which removes their transactivation domain and generates the respective repressor forms that translocate to the nucleus to repress target gene expression [174]. In addition to this process, full-length GLI proteins are kept inactive through their interaction with SUFU, which sequester GLI proteins in the cytoplasm. In the presence of HH ligand, its binding to the PTCH receptor relieves the inhibition of SMO. Activated SMO then blocks the proteolytic cleavage of GLI proteins and promotes their translocation into the nucleus where they induce the expression of HH target genes by also replacing their repressor form [455]. Interestingly, during differentiation the transcript level of *GLI2* and *GLI3* was strikingly upregulated in GLI2$^{+/-}$-derived EPs. By contrast, the expression of *GLIS3*, which has been shown to directly regulate the early endocrine marker *NGN3*, was reduced in mutant EP cells. In addition, the levels of genes upstream of *NGN3*, such as *PDX1*, *FOXA2*, *SOX9* and *HNF1B,* were impaired in patient-like EPs in comparison to WT. Overall, the observed defects in endocrine differentiation affected not only β-cell differentiation but also other endocrine lineage-specific genes, including α-cell and δ-cell-specific markers. This is in line with defects in NGN3 and a decreased pool of endocrine progenitors.

During pancreas development and β-cell function a precise spatiotemporal HH regulation appears to be important. In mice, repression of HH signalling is required for pancreas fate specification and cell differentiation [173,446]. When homozygous GLI2$^{+/+}$ iPSCs were differentiated in the presence of the HH inhibitor SANT-1, the cells could further advance in their differentiation status, suggesting that modulation of the HH pathway might rescue, to some extent, the endocrine phenotype of the patient-like cells. However, these findings need to be further confirmed, because the RNA-Seq highlighted dysregulation of multiple signalling pathways, like Wnt and FGF signalling, in GLI2$^{+/-}$. Also, distinct signalling pathways are modulated *in vitro* in the various differentiation protocols. For instance, Rezania et al. uses the selective BMP signalling inhibitor LDN-193189 in combination with HH inhibition to progress from definitive endoderm to endocrine progenitor stage [204]. In contrast, Russ et al. applies LDN at later stages, after reaching the pancreatic progenitor stage [205].

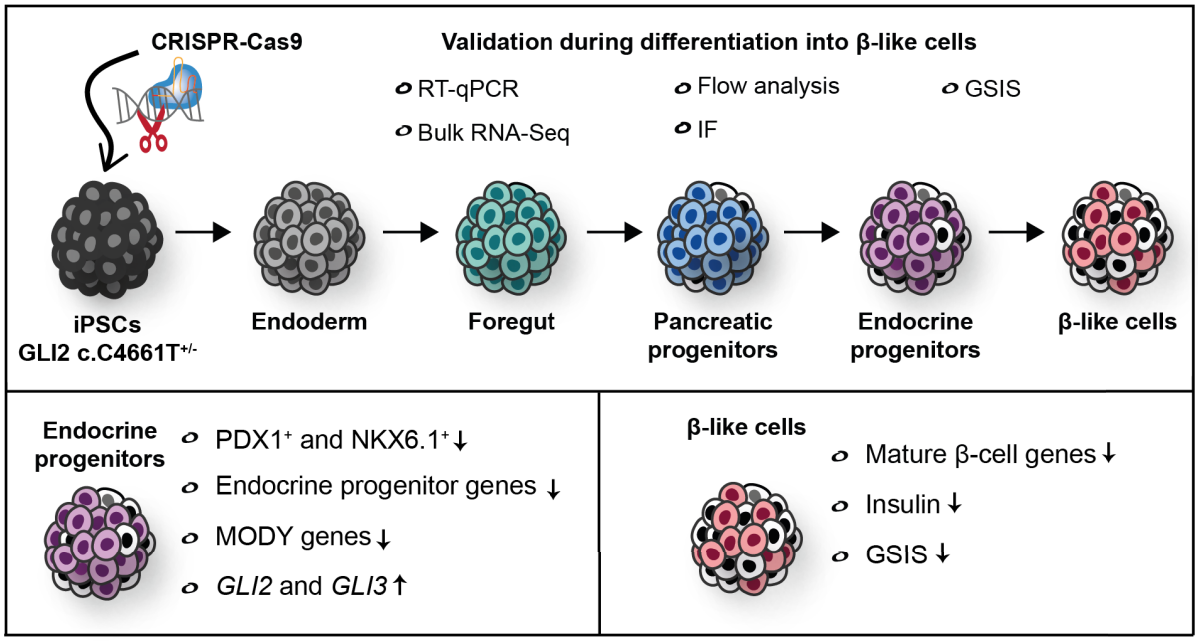

Fig. 30: The novel patient variant c.C4661T impairs differentiation into β-like cells. Schematic representation of the phenotype observed in a newly established GLI2 c.C4661T disease model. The patient variant c.C4661T in GLI2 is introduced into iPSCs using CRISPR-Cas9 genome editing. By combining gene-editing with directed differentiation the role of the novel variant in GLI2 is studied during β-cell development. As determined by RT-qPCR, IF, RNA-Seq and GSIS the differentiation of heterozygous GLI2 c.C4661T iPSCs into endocrine progenitors and β-like cells is impaired.

Previous studies have highlighted that the primary cilium, a microtubule-based organelle present in almost all mammalian cells, plays a prominent role in modulating HH signalling [456–458]. Moreover, it has been reported that localization of GLI2 at the tip of the cilium is required for its activation and nuclear translocation. More recently, Kluth and

colleagues demonstrated that dysregulation of cilia-associated genes may increase the susceptibility for T2D [459]. Since the ciliary localization of GLI2 is important for cilium-dependent activation of SHH signalling, I am currently investigating the localization of WT and mutant GLI2 protein in endocrine progenitor cells. Beside the presence of the cilium, their length and distribution are important for proper SHH signalling [460]. These factors might further contribute to the impaired differentiation of GLI2$^{+/-}$-iPSCs into β-like cells and are in focus of future investigations.

Normal β-cells metabolize glucose essentially via aerobic glycolysis, wherefore the expression of the lactate/pyruvate transporter MCT1, encoded by the *Slc16a1* gene is repressed. Failure of this repression is associated with a rare genetic disease called exercise-induced hyperinsulinism due to inappropriate insulin release [229,461–463]. During β-cell differentiation repression of these so called β-cell disallowed genes, *Slc16a1* and *LDH*, is therefore crucial. Notably, in endocrine progenitors derived from GLI2$^{+/-}$-iPSCs the expression of *Slc16a1* was upregulated. Together with the reduced glucose-responsiveness of mutant β-like cells this data suggests that *GLI2* c.C4661T may also alter β-cell functionality. Further studies are necessary to address this aspect in more details.

Overall, the work of this thesis suggests that GLI2 regulates β-cell development and function through transcriptional regulation of a set of genes, whose dysregulation might contribute to the pathogenesis of monogenic and common types of diabetes. Future studies will be essential to dissect the mechanism by which the novel variant c.C4661T in *GLI2* contributes to diabetes. The patient phenotype of *GLI2* c.C4661T may also be influenced by yet unidentified factors. Therefore, the established platform in the present study may be further utilized to screen unbiased chemicals for novel drug discovery that might improve patient-like iPSC phenotype.

5.4 Novel identified extrinsic factors improve the efficiency of β-cell differentiation

A new hope for the treatment of various degenerative diseases is stem cell-based therapy. In particular for T1D, stem cell-based cell replacement therapy represents a possible cure being the disease caused by the loss of a single cell type, which does not need to be transplanted back in its endogenous site to perform its function. The Edmonton protocol in 2000 established proof-of-principle. Here, islet cells isolated from healthy cadavers are transplanted into the liver of T1D. This protocol can lead to insulin independence in patients for several years [345]. Recently several groups reported the differentiation of β-like cells

starting from human PSCs [204,205,348]. However, the efficiency in generating β-like cells is still low and varies between different groups and cellular sources. Moreover, the response to glucose in iPSC-derived β-like is very limited due to their immature nature. It is therefore likely that current protocols still lack critical signalling factors, which are required for the efficient endogenous differentiation of mature insulin-secreting β-like cells *in vivo*. In the context of β-cell differentiation, extrinsic factors play a major role in controlling cell fate and function. Here, through genetic studies in the mouse (SLIT3) and unbiased high-throughput chemical screening (HC toxin) in zebrafish, I characterized two novel identified extrinsic factors which showed conserved role in enhancing human endocrine differentiation or β-cell maturation/function, respectively.

High-throughput screening (HTS) of chemical libraries has become a critical tool in basic biology and drug discovery. However, HTS in murine models is not feasible due to high costs per animal, time and space management. Oppositely, drug testing in small animals, such as zebrafish or *Xenopus* embryos, allows to combine large-scale therapeutic screening rates with the complex physiology of a whole organism [464]. In the context of diabetes, a few zebrafish screens mainly focusing on β-cell mass, β-cell regeneration or gluconeogenesis have been published [465,466]. However, so far none of these *in vivo* screens targeted β-cell maturation/function. In collaboration with the laboratory of Prof. Dr. Didier Y. Stainier, who identified several novel regulators of pancreatic endocrine development in a zebrafish screen, I validated the conserved role of HC toxin in human β-cell differentiation [419]. HC toxin is a cyclic tetrapeptide originally identified as a host-selective toxin from the maize pathogenic fungus Cochliobolus (Helminthosporium) carbonum and inhibits class I HDAC enzymes in mammalian cells [467,468]. So far, HDAC inhibitors have not been used in β-cell differentiation protocols. My findings suggest that the addition of HC toxin can efficiently induce the maturation of β-like-cells *in vitro*. Interestingly, other studies reported that HDAC inhibitors, like trichostatin A, protect β-cells from apoptosis under stress situations such as cytokine treatment [469]. Therefore, HDAC inhibitors have emerged as potential new therapeutics for diabetes. However, these studies did not examine the expression of mature β-cell markers, including *PDX1* or *MAFA*. This is important during the progression of diabetes, where a reduction in β-cell mass is accompanied by β-cell dedifferentiation, including reduced expression of key β-cell TFs and upregulation of β-cell disallowed genes (e.g. *LDHA*) or pancreatic progenitor genes (e.g. *NGN3*) [470,471]. In another study, HC toxin was shown to enhance glucose uptake and metabolism in the muscle [472,473]. Although, the authors focused on mechanisms downstream of insulin, the observed protective effect of HDAC inhibitors could be a combination of an increase in insulin secretion by β-cells together with a higher glucose uptake by target tissues. This would be in line with my findings that HC toxin

supports β-cell maturation *in vitro*. Further studies are needed to understand the mechanism of action of HC toxin and other HDAC inhibitors.

In summary, my results strongly suggest that an *in vivo* strategy in zebrafish is able to identify compounds with possible conserved activities in humans. Notably, the Alk5 inhibitor, which was also identified in the zebrafish screen, is already applied in current β-cell differentiation protocols. Therefore, the combination of a whole-organism screen with a human PSC-derived pancreatic β-cell differentiation model represents an ideal platform to test and validate new chemicals for human β-cell development.

Current pancreatic differentiation protocols are based on 3D culture conditions to promote differentiation of PSCs and improve the functionalities of *in vitro* generated β-like cells. Several transplantation studies have demonstrated the improvement of differentiation of immature β-like cells or pancreatic progenitors into mature β-like cells *in vivo* [204,205,348,352]. Moreover, recent studies have started to provide insights into how the 3D microenvironment enhances endocrine differentiation in the developing pancreas [170,474–477]. Therefore, the *in vivo* microenvironment is important for pancreatic β-cell development and maturation.

The main component of the embryonic microenvironment is the mesenchyme. Recent single-cell transcriptome analysis has underscored an high degree of heterogeneity within this compartment [478]. Yet is unknown if the pancreatic microenvironment comprises functionally distinct subsets of cells that create distinct niches [43]. Recent work in the Spagnoli laboratory identified two subsets of pancreatic mesenchymal cells in mice, which exert distinct functions during pancreatic development through deposition of extracellular matrix proteins and secretion of signalling factors. Among the different soluble factors released by the pancreatic mesenchyme, the Slit3 ligand was detected as particularly abundant. Slit3 is the ligand of Robo (Cozzitorto et al., under 2nd revision).

The Robo/Slit signalling pathway is primarily known for its role in axon guidance during nervous system development. Recently, this pathway has been involved in many other physiological and pathological processes, such as organogenesis, stem cell regulation and cancer [420,421,479]. ROBO and SLIT have been previously found in human pancreatic tumour microenvironment, but it is still unknown if they are present in the microenvironment surrounding human embryonic pancreas [420,422]. Also, recent work from the Spagnoli laboratory identified the Robo/Slit signalling pathway as a potent gatekeeper of pancreatic identity [423]. Results from my thesis work further suggest the functional conversation of mesenchymal SLIT factors in human endocrine differentiation. The addition of human recombinant SLIT3 to the pancreatic differentiation protocol promoted the formation of endocrine progenitors and maturation of β-like cells. Further investigations are ongoing to

define in more detail the specific window of action (e.g. treatment at different time points), dose response, and impact on β-cell functionality. Furthermore, I am testing other mesenchymal factors that were identified *in vivo* in the mouse embryonic mesenchyme. Ultimately, an in-depth insight into the complexity of the *in vivo* pancreatic microenvironment will help in optimizing current protocols for differentiating human PSCs into β-like cells.

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8 ABBREVIATIONS

AFP	α-fetoprotein
ABCC8	ATP-binding cassette sub-family C member 8
Acetyl-CoA	Acetyl coenzyme A
ActRIIA	*Activin receptor type IIA*
ActRIIB	*Activin receptor type IIB*
APC	Adenomatosis Polyposis Coli
APPL1	Adapter protein containing PH domain
Arx	*Aristaless-related homeobox*
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
BLK	*B lymphocyte kinase*
BME	Basal Medium Eagle
BMP	Bone Morphogenetic Protein
Bmpr1a	*BMP receptor 1A*
BP	Biological Process
bp	base pair
bZIP	Basic Leucine Zipper Domain
c-Myc	Myelocytomatosis oncogene
Ca	calcium
CADD	Combined Annotation Dependent Depletion
Cas9	CRISPR associated protein 9
CEL	*carboxyl ester lipase*
Celsr2	*Cadherin EGF LAG seven-pass G-type receptor 2*
Chip-seq	chromatin immunoprecipitation sequencing
CK-1	*Casein kinase-1*
Cpa1	*Carboxypetidase A1*
CPEP	C-peptide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CRISPRi	CRISPR interference
CSF1	Colony Stimulating Factor 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
d	day
D/V	Dorsal-Ventral
DE	Definitive Endoderm

DHH	Desert hedgehog
DKK	Dickkopf
dl	deciliter
Dll	Delta-like
DNA	Deoxyribonucleic acid
DNML1	Dynamin-1-like protein
DNMT3a	DNA methyltransferase 3A
DOCK1	Dedicator of cytokinesis protein 1
DOX	Doxycycline
dp	dorsal pancreas
DSB	double-strand break
Dsh	dishevelled
E	Embryonic day
e.g.	exempli gratia
E8	Essential 8 Medium
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor receptor
EN	Endoderm
EOMES	Eomesodermin
EP	Endocrine progenitor
ESCs	Embryonic Stem Cells
et al.	et alia
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
FACS	Fluorescence-Activated Cell Sorting
FAD	flavin adenine dinucleotide
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
Fgfr	Fibroblast Growth Factor Receptor
Foxa1	*Forkhead box A1*
Foxa2	*Forkhead box A2*
FoxO1	Forkhead Box O1
Fzd	Frizzled
G6P	Glucose-6-phosphotase
GAD	Glutamic acid decarboxylase
GCK	Glucokinase
GDM	Gestational diabetes mellitus
GFP	Green fluorescent protein
GLC	Glucagon
GLI	Glioma-associated oncogene homolog
GLIS3	GLIS Family Zinc Finger 3
GLUT2	Glucose transporter 2
GO	Gene Ontology

GP2	Glycoprotein 2
GRG3	Groucho-related protein 3
GSIS	Glucose-stimulated insulin secretion
GSK3β	Glycogen synthase kinase 3
Gw	weeks of gestation
GWAS	Genome-wide association studies
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDR	Homology-directed repair
Hes1	*Hairy and enhancer of split*
Hex	*Hematopoietically expressed homeobox gene*
HH	Hedgehog
HLA	Human leukocyte antigen
Hnf1b	HNF1 homeobox B
Hoe	Hoechst
Hox	Homeobox
IA	Tyrosin-Phosphatase
iCRISPR	inducible CRISPR
IDE	Inducer of definitive endoderm
IF	Immunofluorescence
IHH	Indian Hedgehog
INS	Insulin
Insm1	*Insulinoma-associated 1*
iPSCs	induced Pluripotent Stem Cells
ISH	In Situ Hybridization
Isl1	*Islet1*
JMJD3	Jumonji domain-containing protein D3
KCNj11	ATP-sensitive inward rectifier potassium channel 11
KLF11	*Kruppel Like Factor 11*
KMT	Lysine methyltransferases
KO	knock-out
LEF	Lymphocyte enhancer factor
LRP	Lipoprotein receptor-related protein
LSD1	Lysine-specific demethylase 1
MafA	*v-Maf musculoaponeurotic Fibrosarcoma oncogene family, protein A*
MafB	*v-Maf musculoaponeurotic Fibrosarcoma oncogene family, protein B*
MEF2	myocyte enhancer factor 2
MNX1	*Motor Neuron And Pancreas Homeobox 1*
MODY	Maturity-Onset Diabetes of the Young
MPCs	Multipotent pancreatic Progenitor Cells
mRNA	messenger Ribonucleic Acid
n	number
ND	Not Determined

NDM	Neonatal diabetes
NeuroD1	*Neurogenic differentiation 1*
Ngn3	*Neurogenin-3*
NHEJ	non-homologous end joining
Nkx2.2	*Nkx2 homeobox 2*
Nkx6.1	*Nkx6 homeobox 1*
OCT4	*Octamer-binding transcription factor 4*
OD	Optical Density
Onecut1	*One cut domain 1*
PAM	Protospacer adjacent motif
Pax4	*Paired box 4*
Pax6	*Paired box 6*
PCP	planar cell polarity
PCR	Polymerase Chain Reaction
PCSK1	*Proprotein convertase subtilisin/kexin*
PDNM	Permanent NDM
Pdx1	*Pancreatic and duodenal homeobox 1*
PFA	Paraformaldehyde
Polyphen-2	Polymorphism Phenotyping v2
PP	Pancreatic polypeptide cells
PrDOS	Protein DisOrder prediction System
PRMTs	Arginine methyltransferases
Prox1	*Prospero homeobox 1*
PSCs	Pluripotent Stem Cells
Ptch	*Patched*
Ptf1a	*Pancreatic specific Transcription Factor 1a*
PTMs	Posttranslational histone modifications
PTPN22	Phosphatase-non-receptor type 22
RA	Retinoic Acid
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
RFX6	Regulatory Factor X 6
RNA-Seq	RNA-sequencing
Robo	Roundabout signaling pathway
rs	refSNP
RT-qPCR	Reverse Transcription-quantitative Polymerase Chain Reaction
SCNT	Somatic cell nuclear transplantation
SDS	Sodium-dodecyl sulfate
SETD7	SET Domain Containing 7
SFRP	Secreted-frizzled-related peptide
sgRNA	single guide RNA
SHH	Sonic Hedgehog
SIFT	Sorting Intolerant From Tolerant
SIRT	Sirtuins

Slit	Slit guidance ligand
SMAD	Small body size Mothers Against Decapentaplegic
Smo	Smoothened
SNPs	Single-nucleotide polymorphisms
SOX	Sex-determining region on Y box
Spop	Speckle-type POZ protein
ssODN	single-stranded oligo DNA
SST	Somatostatin
Sufu	Suppressor of Fused
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TALE	Three-Amino-Acid Loop Extension
TALEN	Transcription-Activator Like Effector Nucleases
TBX3	*T-Box Transcription Factor*
TCF	T-cell factor
TF	Transcription Factor
Tgfbr	Transforming Growth Factor Beta Receptor
TGFβ	Transforming Growth Factor Beta
TGIF	TG-Interacting Factor
TLE3	Transducin-like enhancer protein 3
TNDM	Transient NDM
TSS	Transcription-Starting Site
USD	United States Dollar
VA	vitamin A
VD	vegetal-dorsal
VEGF	Vascular Endothelial Growth Factor
vp	ventral pancreas
WHO	World Health Organization
Wnt	Wingless-type MMTV integration site family
WT	Wild Type
ZFN	Zinc Finger Nuclease
ZnT8A	Zinc transporter 8 autoantibody
β-TrCP	β-transducin repeat containing protein

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